Food Chemistry 483 (2025) 144225

Contents lists available at ScienceDirect

Food Chemistry

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

Variation in composition of storage proteins, minor proteins and amino acids across faba bean cultivars☆

Tianzhen Xiao[a], Ida Schwartz Roland[a], Søren Drud-Heydary Nielsen[a], Thao T. Le[b], Esben Olesen[a], Lotte Bach Larsen[a], Nina Aagaard Poulsen[a,*]

[a] Department of Food Science, Aarhus University, Aarhus 8200, Denmark
[b] AUT Centre for Future Foods, School of Science, Auckland University of Technology, Auckland 1010, New Zealand

ARTICLE INFO

Keywords:
Faba bean
Globulin
Legumin
Vicilin
Amino acids
Proteomics

ABSTRACT

This study aimed to investigate variations in protein profiles and amino acid (AA) compositions in faba bean seeds among 10 different cultivars. The compositions of salt-soluble proteins and AA were identified and quantified by label-free nano LC-MS/MS and LC-MS/MS (Triple Quadrupole), respectively. The protein contents (dry basis) in faba bean seeds were measured using the DUMAS method, ranging from 21.6 % to 26.8 %. By nano LC-MS/MS, 103 unique proteins were identified under point mutation monitoring analysis and of these, 61 proteins were quantified by label-free quantification. In all cultivars, vicilin and legumin were the most abundant proteins, respectively, varying from 16 to 39 % and 12 to 34 % of total protein content (*w*/w). The relative abundance of minor proteins like Bowman-Birk proteinase inhibitors was quantified to constitute around 1 % of the total protein content (*w*/w). There were 400 mg essential amino acids (EAAs) detected per gram protein, where histidine showed the highest concentration (64–82 mg/g protein) in all faba beans.

1. Introduction

Faba bean (*Vicia faba* L), also known as horse bean or broad bean, has a long history of cultivation, but has largely been overlooked as a food crop compared to soy, pea and chickpea in the past decades. Legumes can fertilize the soil by fixing atmospheric nitrogen and yield considerable amounts of sustainable proteins in their seeds (Jensen et al., 2010). Among legumes, faba beans and peas are cold-season crops that can adapt to low temperatures (Stoddard et al., 2006). Peas are widely applied in food industries, whereas faba beans are primarily utilized as animal feed in Europe, although they exhibit higher yields compared to peas (Koivunen et al., 2016; Millar et al., 2019). Faba bean seeds contain around 25 % protein of the dry weight, and the major proteins are storage proteins. The storage proteins are synthesized in the early stages of seed development and are accumulated in discrete deposits called protein bodies in the cotyledon during final maturity (Multari et al., 2015; Warsame et al., 2022). These proteins serve mainly as reserves for nitrogen and AAs for the plant embryo (Shewry et al., 1995), whereas other minor proteins hold metabolic and structural functions during seed development. These include antinutrient proteins like Bowman-Birk proteinase inhibitors (BBI) and lectins, which can lead to decreased nutrient availability after human consumption by binding to specific proteases or carbohydrates, respectively (Liener, 1994). Faba bean has been profoundly investigated about nutrient contents and possibilities for plant breeding in Europe (Labba et al., 2021; Pulkkinen et al., 2016; Warsame et al., 2020). However, knowledge of variation in protein profiles among faba bean cultivars is far from complete.

According to Osborne classification, proteins can be classified as water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, weak acid-soluble glutelins and finally remaining insoluble proteins (El Fiel et al., 2002; Griffiths & Lawes, 1978). Globulins, as the most abundant fraction of faba bean storage proteins, account for more than 80 % of the total proteins (*w*/w) in faba seeds (Warsame et al., 2018). Depending on their sedimentation coefficients, faba bean globulins are designated as 11S legumin or 7S vicilin-like proteins (vicilin and convicilin types). Legumin is a hexameric protein with a molecular weight (MW) of 360–400 kDa, assembled by six subunits sharing similar structures. These subunits can be further grouped into legumin type A, legumin type B, and high molecular weight subunits (Müntz et al., 1999). Legumin types A and B with MW of 60 kDa are the most common

---

☆ This article is part of a Special issue entitled: '3rd NIZO Plant Protein' published in Food Chemistry.

* Corresponding author.
E-mail address: nina.poulsen@food.au.dk (N.A. Poulsen).

https://doi.org/10.1016/j.foodchem.2025.144225
Received 9 December 2024; Received in revised form 27 March 2025; Accepted 4 April 2025

subunits of the faba bean legumins and are composed of two disulfide-linked polypeptides (acidic 40 kDa α-chain and basic 20 kDa β-chain) (Croy et al., 1980). The high molecular weight subunits of 75–80 kDa are very low abundant in faba beans (Fuchs & Schubert, 1995). Legumin type A and B are encoded by two distinct gene subfamilies, leading to significant protein heterogeneity among varieties and even within single genotypes of the same variety (Shewry et al., 1995; Wobus et al., 1986). Previously, 29 pairs of legumin α-chain and β-chain were detected in 1- and 2D gels of purified faba bean legumin (Tucci et al., 1991). The MWs of these α- and β-chains ranged from 20 to 60 kDa, and their isoelectric points (pI) varied from 6.2 to 7.4. The 7S vicilin-type proteins, including vicilin and convicilin, are trimerics, which are assembled by three subunits with MWs of either 50 or 70 kDa. Vicilin, with MW ranges from 150 to 190 kDa, is composed of either two 50 and one 70 kDa chains, or two 70 kDa and one 50 kDa chains (Müntz et al., 1999). Convicilin, with a mass of 220–290 kDa, was first defined in peas and subsequently in other legumes including faba beans. It is composed of three 70 kDa chains, including a highly charged hydrophilic N-terminal extension (O'Kane et al., 2004; Sáenz de Miera et al., 2008).

The BBI and lectins both belong to the water-soluble albumin fraction of faba beans (Park et al., 2010), and are often co-extracted with the globulins. The BBI, with a mass of approximately 8 kDa, consists of two homologous domains, that can inhibit both trypsin and chymotrypsin, independently and simultaneously (Asao et al., 1991). Faba bean lectins, also called favins, are dimers that contain subunits composed of an α-chain (7 kDa) and a β-chain (20 kDa). Both chains contain binding sites for metal ions and specific oligosaccharides (Reeke Jr. & Becker, 1986).

As the fundamental unit of protein, AA have been widely studied to evaluate the protein quality of food legumes (Liu et al., 2017; Mustafa et al., 2007). Legume seeds are generally deficient in sulfur-containing AA (S-AA) methionine and cysteine, but rich in lysine, which is complementary to cereals that are rich in S-AA, but limited in lysine (Geil & Anderson, 1994; Iqbal et al., 2006). As methionine is an essential amino acid (EAA), it must be obtained from the diet, whereas cysteine as a non-essential amino acid (NEAA) can be synthesized in the body. The composition of S-AA varies distinctly in legumin and vicilin, leading to different functionalities of faba bean globulins. In faba bean, methionine has only been detected in legumin type A among the globulins (Gueguen & Cerletti, 1994; Müntz et al., 1999). The 7S globulins (vicilin and convicilin) from faba bean were found to have higher solubilities at pH 5–6, and higher emulsifying properties compared to the 11S globulins (legumins, Kimura et al., 2008). Thus, the ratio of 11S globulins to 7S globulins (the L/V ratio) was used as an index of legume protein quality (Husband et al., 2024; Shi & Nickerson, 2022). Warsame et al. (2020) found that the L/V ratio in faba bean varied from 1 to 3 across thirty-five genetically diverse faba bean genotypes using size exclusion high-performance liquid chromatography. Generally, the L/V ratio is susceptible to genotype, growth conditions, and protein extraction processes (Müntz et al., 1999). Therefore, the L/V ratio in faba bean across cultivars needs to be further investigated.

This study aimed to investigate and identify variations of faba bean seed protein profiles across 10 different Danish-grown cultivars by applying sensitive proteomics using nano LC-MS/MS timsTOF Pro II that enables relative quantification of protein without using label agents. The use of LC-MS/MS for protein identification and quantification in the complex matrix has been well established in previous studies (Chelius & Bondarenko, 2002; Vreeke et al., 2023), where the methodology of peptide release and protein quantification was exquisitely developed. The *Vicia* protein library from UniProt was filtered and used for protein annotation. Moreover, the AA composition of faba bean was determined after acid hydrolysis by LC-MS/MS (QQQ) using the AccQtag reagent for derivatization to improve efficiency (Diep et al., 2020; Salazar et al., 2012).

2. Materials and methods

Dried faba bean (*Vicia faba* L) seeds from 10 different cultivars were collected from individual producers and plant breeders in multiple regions of Denmark. The cultivars included Apollo, Birgit, Capri, Daisy, Fuego, GL Arabella, Granit, Lynx, Stella, and Tiffany, which were harvested between 2021 and 2022. Before analyses, the dry seeds were milled into fine powders using a rotor mill with a sieve mesh size of 0.5 mm (Retch, Germany, ZM 200Ultra Centrifugal Mill).

2.1. Protein determination and extraction from seeds

The protein contents of raw faba beans were determined following the AACC combustion method 46–30.01 (AACC, 2025) using an automated Dumatherm N Pro protein analysis system (Gerhardt Gmbh & Co, Germany) with a protein conversion factor of 5.4 (Mosse, 1990). The protein contents were analyzed in biological triplicate. The salt-soluble proteins of faba beans were extracted according to Mertens et al. (2012) with some modifications. In brief, 100 mg of raw faba bean flour was mixed with 1 mL of 150 mM PBS buffer (pH 7.4). The mixture was shaken at 1000 rpm for 30 min and centrifuged at 20000 ×*g* for 20 min at room temperature (RT). The supernatant was collected as protein extracts for further analysis. The extraction was conducted in biological triplicates. According to Mertens et al. (2012), the collected supernatant is a mixture of salt-soluble globulins and water-soluble albumins. Thus, both faba bean globulins and some albumins are expected to be extracted by PBS buffer, whereas minor fractions of prolamins and glutelins in faba beans are excluded by this extraction. Extracts were stored at −80 °C until further analysis.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts from 10 faba bean cultivars

The protein profiles of the 10 cultivars were analyzed using 1D SDS-PAGE under reducing conditions according to Knudsen et al. (2024) with minor modifications. Briefly, 2 mg/mL protein extract was mixed with sample buffer (20 mM Tris, 2 % SDS, 20 % Glycerol, Bromophenol blue) at a ratio of 1:1 and heated at 100 °C for 5 min. The mixture was centrifuged at 10000 ×*g* for 1 min before 15 μL of each supernatant was loaded to the wells of Criterion TGX Precast Protein Gel (Bio-Rad, CA, USA). The PageRuler Plus Prestained Protein Ladder (5 μL) with MW ranging from 10 to 250 kDa (Thermo Scientific, USA) was used as the marker. The gel was run in the Criterion Cell at 200 V for 35 min. Subsequently, the gel was fixed with a solution containing 50 % ethanol and 8 % phosphoric acid for 2 h and stained with Coomassie Blue solution for 1 h followed by destaining with MilliQ water. The gel was imaged using Image Lab (Bio-Rad Laboratories, CA, USA).

2.3. 2D SDS-PAGE and MALDI-TOF identification of protein spots

For 2D gel and protein identification, protein extraction from the cultivar Fuego was analyzed according to Jensen et al. (2012) with minor modifications. Briefly, the extracted proteins were diluted to approximately 1 mg/mL with lysis buffer (7 M urea, 2 M thiourea, 1 % dithioerythritol (DTE), 40 mM lysis base buffer pH 7.5) and shaken for 1 h at RT followed by a short centrifugation. Then 200 μL of supernatant was transferred to the lane and covered by 11 cm IPG strips (pH 3–10) (Bio-Rad) using active rehydration in a Protean IEF Cell (Bio-Rad). After running the 1st dimension, the strip was transferred to a glass tube with 5 mL equilibration buffer I (65 mM DTE) for 15 min incubation, and another 15 min incubation using equilibration buffer II (270 mM iodoacetamide (IAA)) before loading on the gel. After running, the gel was fixed, stained and imaged at the same conditions as used for the 1D gel.

The gel spots were cut out from the 2D gel and divided into different batches for digestion according to the intensity of the spots. The gel spots were cut into small pieces and placed in 1.5 mL Eppendorf tubes for overnight destaining using 70 μL of 50 % acetonitrile (ACN). Then the gel pieces were reduced using 10 mM DTE in 0.1 M NH_4HCO_3 at 50 °C for 45 min, and alkylated with 55 mM IAA in 0.1 M NH_4HCO_3 at RT for 30 min in the dark. After washing with 20 μL 50 % ACN, 20 μL 100 % ACN, 20 μL 0.1 M NH_4HCO_3 and 30 μL 100 % ACN, respectively, the samples were resuspended in 10 μL of 12.5 ng/μl trypsin in 50 mM NH_4HCO_3 on ice for 45 min. The excess trypsin solution was removed and 50 mM NH_4HCO_3 was added. The samples were incubated at 37 °C overnight.

The digests were acidified using 10 % TFA and desalted using in-house manufactured columns. The columns were made of clean 20 μL tips filled with tiny amounts of C18 membrane material. The column was activated by 100 % ACN and washed by 0.1 % TFA. The digested peptides were loaded on the column and washed by 0.1 % TFA. Then the digested peptides were eluted using 5 μL α-cyano-4-hydroxycinnamic acid (CHCA) in 70 % ACN, 0.1 % TFA, and loaded on the MALDI target plate. The spectrum from MALDI-TOF was obtained and annotated by FlexControl and FlexAnalysis software, respectively. The resulting peptides were derived from digests of 2D gel spots corresponding to faba bean seed proteins. The mass fingerprint of these peptides was searched against an in-house faba bean protein library derived from Uniprot, which was filtered for faba bean proteins to enhance annotation accuracy. The search was conducted through the Mascot search engine, which generated a score to evaluate the confidence in the identification of a protein match. To evaluate the match, the score was compared to a threshold determined by statistical significance (*p*-values, FDR), scoring models (ion scores, *E*-values), and peptide matching criteria. In this study, the threshold score was 40 and proteins with scores above 40 were considered as significant.

2.4. Nano LC-MS/MS timsTOF pro II proteomics on protein extracts from 10 faba bean cultivars

2.4.1. In solution digestion

For nano LC-MS/MS proteomic analysis, the samples were prepared according to Thesbjerg et al. (2024) with minor modifications. Briefly, the protein extraction was diluted prior to the in-solution digestion. Diluted protein samples (15 μg protein) were denatured by 6 M urea and reduced by 10 mM DTE at RT for 2 h, followed by protein alkylation using 20 mM IAA. After 30 min of incubation at RT in dark conditions, ice-cold trypsin/LysC mix (Promega™, Mass Spec Grade, USA) was added to each sample with an enzyme/protein ratio of 1:20. All samples were then incubated for 3–4 h at RT in darkness. This was followed by the addition of six-fold 20 mM triethylammonium bicarbonate (TEAB) and all samples were incubated overnight at RT in darkness. The digested samples were then desalted using Pierce Peptide Desalting Spin Columns (Thermo Fisher Scientific, Waltham, Massachusetts, USA), following the manufacturer's instructions. After desalting, the eluates containing peptides were dried using Speed Vac (Genevac EZ-2plus, BioLab, Ipswich, UK) and resuspended to 40 μL 0.1 % formic acid (FA). The in solution digestion was conducted in technical triplicates. All samples were loaded to 10 kDa filters before LC-MS/MS analysis to remove contaminants.

2.4.2. Proteome analysis by nano LC-MS/MS timsTOF pro II

Tryptic peptides from faba bean proteins were separated on a nanoElute LC system (Bruker, Solna, Sweden), with a captive-spray ionization source attached to a trapped ion mobility spectrometry time-of-flight (timsTOF Pro II, Bruker, Bremen, Germany) mass spectrometry instrument according to Jiménez-Munoz et al. (2024). A volume of 10 μL of each resolubilized tryptic digest was loaded onto a C18 PepSep Fifteen column (1.9 μm, 150 mm, 75 μm, Bruker). The column temperature was set to 50 °C. Peptides were eluted using 0.1 % FA in water (solvent A) and 0.1 % FA in 100 % ACN (solvent B) with a flow rate of 400 nL/min. The 41 min gradient consisted of 2–17 % solvent B for 22 min, 17–26 % solvent B for 9 min, 26–37 % solvent B for 5 min, 37–95 % solvent B for 1 min and then 95 % solvent B for 4 min. Spectra were collected in positive ionization mode with data-dependent acquisition using parallel accumulation-serial fragmentation. The mass spectrometer was set to scan masses between 100 and 1700 *m/z*.

2.4.3. Data analysis

For MALDI-TOF identification, a *Vicia faba* library of 602 entries was extracted from UniProt. However, for the more sensitive nano LC-MS/MS analysis, the broader taxonomic genus, *Vicia,* was used to broaden the identification as the *Vicia faba* library is somewhat limited. This resulted in a *Vicia* library with 2035 entries. The acquired spectra data were extracted with variable modifications and searched against *Vicia* library (Uniprot, 2035 entries) using the software PEAKS (Bioinformatics Solutions Inc., Waterloo, Canada). Parameters for library searching were set as follows: parent ion mass error tolerance 20 ppm, fragment ion mass error tolerance 0.1 Da, precursor ion mass search type monoisotopic, enzyme trypsin, up to 3 missed cleavage per peptide, digest mode specific, max variable modifications per peptide 3. The variable post-translational modifications (PTMs) included carbamidomethylation, deamidation, acetylation (N-term), phosphorylation, oxidation (M), pyro-glu (Q, E). The false discovery rate was 1 %. The relative abundance of identified proteins was quantified by label-free quantification, where the peak area of the top 3 unique peptides assigned to the protein annotation was calculated to represent the relative abundance of the protein. The SPIDER function of PEAKS was used to allow point mutations.

2.5. AA analysis

The AA composition was quantified by LC coupled with a Triple Q MS (Santa Clara, CA, USA) after acid hydrolysis. Briefly, 15 mg raw faba bean flour from each cultivar was mixed with 5 mL 6 M HCl in a glass vial and flushed with N_2 to avoid oxidation. The glass vial was transferred to an oven and incubated at 110 °C for 22 h and subsequently, cooled and filtered through Whatman filter paper. Then, 200 μL of the solution was transferred to an Eppendorf tube and the HCl was evaporated in a vacuum evaporator at 75 °C for 2 h. The dried samples were redissolved in 1 mL of phosphate-citrate buffer (pH 2.2), and 40 μL was taken out and derivatized using AccQTag reagent as previously described (Roland et al., 2024). The samples were run in technical triplicates and the LC-MS analyses were conducted as described by Roland et al. (2024).

2.6. Statistical analysis

Data was assessed by one-way analysis of variance (ANOVA) with Turkey's post-hoc multiple comparison test using Minitab (version 19.1). The level of statistical significance was determined at $p < 0.05$.

3. Results and discussion

3.1. Variation in protein contents across different faba bean cultivars

The protein contents among the 10 faba bean cultivars were significantly different (Table 1). Cultivar Lynx had the highest dry basis total protein content (26.81 % *w*/w), whereas Apollo had the lowest (21.60 %). The remaining 8 cultivars were comparable in protein contents with an average of 25.05 % (± 1.36 %) without statistical significance. This result was consistent with the findings of Makkar et al. (1997), where no significant difference was found between 6 white-flowering and 6 colour-flowering faba bean cultivars. For cultivars that were also studied in a previous Swedish study, such as Lynx, Fuego and Stella, the protein contents were lower than our results, indicating that faba bean protein contents can be affected not only by cultivar but also by growing conditions (Labba et al., 2021).

Table 1
Protein content and amino acids composition of 10 faba bean cultivars.

Cultivar		Apo	Bir	Cap	Dai	Fue	Gla	Gra	Lyn	Ste	Tif
	Protein (%)	$21.60^{\pm 0.46}$	$25.63^{\pm 0.33}$	$25.35^{\pm 0.3}$	$25.58^{\pm 0.97}$	$25.37^{\pm 0.13}$	$24.46^{\pm 0.16}$	$24.63^{\pm 0.65}$	$26.81^{\pm 1.29}$	$25.59^{\pm 0.61}$	$25.44^{\pm 0.35}$
Amino acid (mg/g protein)	Histidine	67.69	82.41	66.70	65.47	64.44	67.95	72.05	80.04	82.33	66.53
	Phenylalanine	39.32	45.50	40.46	37.72	40.02	40.04	43.17	43.69	48.09	39.63
	Methionine	1.92	2.09	2.02	1.90	2.52	2.21	2.29	2.42	2.58	1.63
	Valine	43.02	49.38	44.46	41.12	44.98	45.59	47.82	47.21	49.77	44.33
	Isoleucine	37.13	42.94	38.59	36.35	38.87	39.18	41.99	41.43	44.87	38.63
	Leucine	54.49	62.06	56.48	52.02	55.21	57.93	61.53	59.96	64.72	56.27
	Threonine	38.55	44.86	38.37	35.99	41.18	39.76	40.88	41.75	43.95	38.26
	Lysine	52.85	57.30	51.27	49.38	56.27	53.74	57.78	53.17	55.91	53.00
	Cystine	1.14	1.86	1.15	1.05	1.10	1.11	0.97	1.25	1.24	0.93
	Arginine	54.14	79.09	66.31	62.98	63.50	76.60	67.76	85.69	81.53	66.40
	Serine	45.61	51.54	46.47	42.61	48.88	47.54	50.04	48.95	54.91	47.05
	Glycine	34.86	38.54	33.77	31.55	37.05	34.60	36.20	36.53	38.92	34.44
	Aspartic acid[a]	99.61	115.32	102.79	94.65	104.63	105.06	113.37	112.23	116.59	104.32
	Glutamic acid[b]	121.83	144.56	127.76	118.26	130.85	131.40	138.24	139.65	146.45	126.46
	b-Alanine	0.36	0.36	0.37	0.31	0.46	0.38	0.40	0.39	0.38	0.35
	Alanine	28.32	31.86	28.50	26.19	29.89	29.46	30.24	30.48	30.12	28.27
	Tyrosine	0.70	1.81	0.71	0.49	0.62	0.77	0.70	0.54	0.92	0.87
	Proline	41.57	48.83	42.43	39.90	44.61	44.54	45.53	46.70	48.51	43.27
	EAAs	389.11	465.63	404.66	382.93	406.99	423.00	435.27	455.36	473.75	404.68
	Total	763.11	900.31	788.61	737.94	805.08	817.86	850.96	872.08	911.79	790.64
Non-protein amino acids	Hydroxy-L-Proline	1.12	1.19	0.99	0.93	1.42	0.94	1.03	1.04	1.12	0.93
	Ethanolamine	1.06	1.08	0.91	0.92	1.10	0.98	1.06	1.07	1.07	0.84
	gamma-Amino-n-butyric acid	0.19	0.18	0.15	0.16	0.41	0.44	0.19	0.22	0.19	0.16
	delta-Hydroxylysine	0.00	0.00	0.09	0.00	0.10	0.10	0.00	0.11	0.00	0.08
	LOrnithine	0.22	0.23	0.24	0.18	0.24	0.26	0.22	0.28	0.21	0.23
	Taurine	0.09	0.00	0.05	0.06	0.10	0.05	0.08	0.10	0.05	0.06

Values are means ±standard deviation.
a Aspartic acid and asparagine combined.
b Glutamic acid and glutamine combined.

3.2. Proteomic analyses

3.2.1. Protein compositions by 1D SDS-PAGE

The salt-soluble faba bean protein profiles are shown in Fig. 1. Overall, the 10 cultivars displayed quite similar protein band patterns. Collectively, 28 protein bands with MW ranging from 11 to 120 kDa (numbered from 1 to 28, Fig. 1) were observed among 10 cultivars. The most intensive bands were observed at MW around 48 (ID 12) and 36

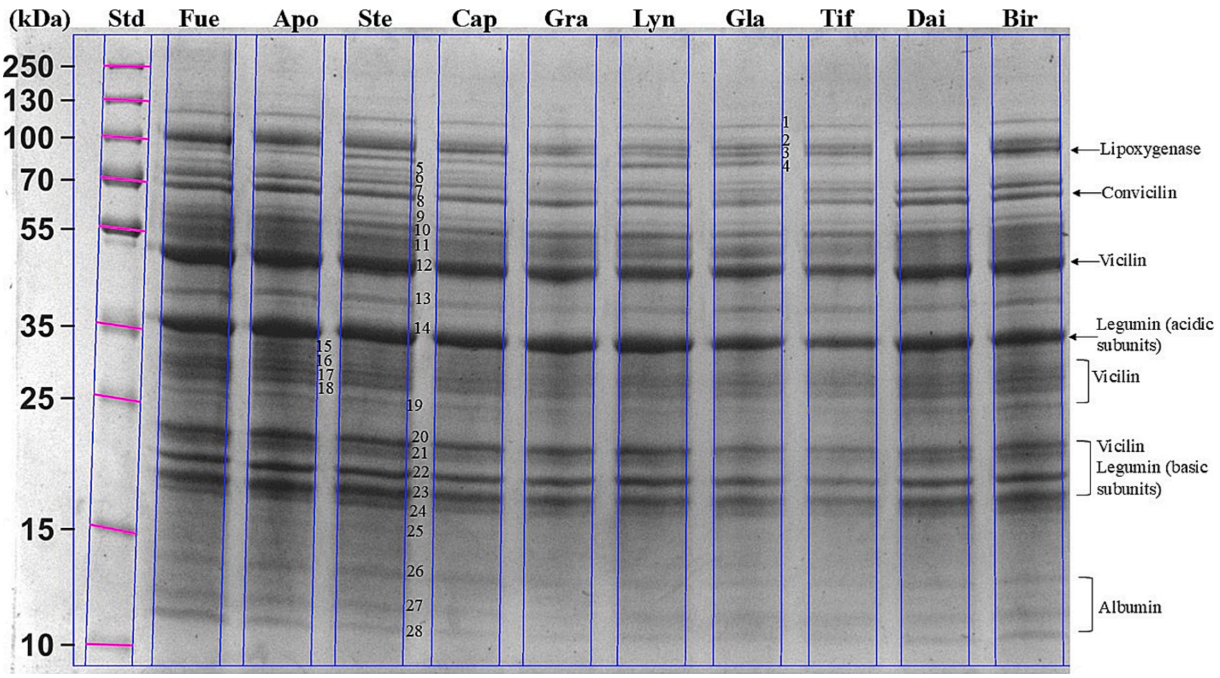

Fig. 1. 1D SDS-PAGE of salt-soluble proteins from 10 faba bean cultivars: Fuego, Apollo, Stella, Capri, Granit, Lynx, Gl. Arabella, Tiffany, Daisy, Birgit under reducing condition and visualized with Coomassie Blue. The protein ladder on the left indicates protein size, and on the right are identified proteins based on previous findings. Std means standard label. Numbers 1–28 highlight the most intense gel bands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

kDa (ID 14), followed by bands between 20 and 25 kDa (bands with ID 20–24). This pattern was consistent with previous studies on faba bean protein profiles on 1D gels, where the most intensive bands were identified as faba bean globulins: vicilin 48 kDa and legumin subunits 36 kDa (Warsame et al., 2020). Earlier studies have shown that the subunits of legumin, namely legumin type A and B with MW of 60 kDa, are composed of α and β chains (roughly 40 and 20 kDa, respectively, dependent on sequence variant and gel system). The α and β chains were linked by disulfide bonds. Thus, there was one band at MW of 55 kDa under non-reducing conditions, but four major bands (with MWs of 36 kDa and 20–25 kDa) at reducing conditions where the disulfide bonds were destroyed (Tucci et al., 1991). This further supported that faba bean legumin subunits are consistently present across cultivars with a major band at 36–38 kDa, and multiple minor bands at 20–25 kDa, as also observed in Fig. 1. Vicilin, being a trimeric protein composed of sulfur-free subunits, has protein bands with major MWs of 70 and 50 kDa. The faba bean vicilin heterogeneity has been reported in 1D gels to be represented as four major bands with MWs of 66, 48, 46 and 43 kDa, in addition to multiple minor bands from 15 to 37 kDa, which can be the result of the intricate PTMs and/or internal proteolysis, adding to protein MW heterogeneity (Tucci et al., 1991). Thus, protein bands with MW of 20–25 kDa (ID 20–24) were regarded as representing combinations of the legumin β chains and of vicilin isoforms, including truncated forms due to proteolysis. The protein band at 70 kDa (ID 8) was ascribed to convicilin, the other 7S globulin found in both pea and faba, with a major single discrete band at 70 kDa (Gatehouse et al., 1981). The protein bands at 100 kDa (ID 3) and 75 kDa (ID 7) have been reported earlier to represent lipoxygenase and heat shock proteins, according to the MS analysis of Warsame et al. (2020). Minor bands with MWs at 27–33 kDa (ID 15–18) could be combinations of fragments of vicilins and legumins due to proteolysis (Tucci et al., 1991). Minor bands between 10 and 15 kDa (ID 26–28) are regarded as proteins belonging to the albumin fraction. In total, the salt-soluble proteins of faba beans were dominated by legumin, vicilin and convicilin as shown in Fig. 1, where consistent patterns were observed across 10 cultivars. Nevertheless, there were six polymorphic protein bands (ID 2, 4, 5, 11, 16, 19) detected in only some cultivars, indicating minor differences in gross protein compositions. These observed variations could be a result of heterogeneity in gene expression or gene mutations across cultivars. It is noted, however, that the complexity of faba bean protein profiles exceeds that suggested by the 1D gel results, as each protein band and intensity represent a mixture of multiple proteins.

3.2.2. Protein identification by 2D SDS-PAGE combined with MALDI-TOF

To further investigate details of faba bean protein composition, the protein extract of cultivar Fuego was subjected to 2D SDS-PAGE, combined with MALDI-TOF for protein identification. In Fig. 2, it is shown that 84 distinctive spots could be observed (spots 1–84). A large proportion of the protein spots had pI values between 5 and 6, and within this range the most intensive spots had MWs of 36–38 kDa (spots 30 and 31), followed by spots with MWs of 48–52 kDa (spots 12 and 13). Furthermore, relatively intense spots with MWs of 20–25 kDa could be observed (spots 49–65), including scattered spots with pI values of 5–6 (spots 9–51 and 61–65). Furthermore, there were some intensive spots at pI around 8 (spots 55–57), indicating different protein isoforms at identical MWs but with different pI values. In addition, there were different horizontal trains of spots with varying intensities (for instance spots 3–7). These are expected to represent different protein isoforms, related either to sequence variation or post-translational modifications, exhibiting complexities in the faba bean proteins, not identifiable by 1D SDS-PAGE.

All protein spots with 84 indicated IDs were excised and subjected to MALDI-TOF, of which, however, only 27 spots resulted in significant identifications. All identified proteins were assigned to globulins, except spot 16, identified as putative sucrose binding protein (Table 2). In accordance with the 1D gel analysis, bands with MWs of 36 and 48 kDa were the most intensive across all cultivars, and the most intensive spots in the 2D gel were found also at positions corresponding to these MWs (spots 30, 31, 12, 13). By MS analysis, spots 30 and 31 were identified as legumin A2, containing 6 methionine. Legumin A1, containing 4 methionine, was also identified in spot 31. Spots 29 and 30, having identical MWs, but different pIs, were both identified as legumin A2, suggesting AAs that they may represent different genetic variants or isoforms. Spots 12,13 and 14, with MWs of 48 kDa, were identified to

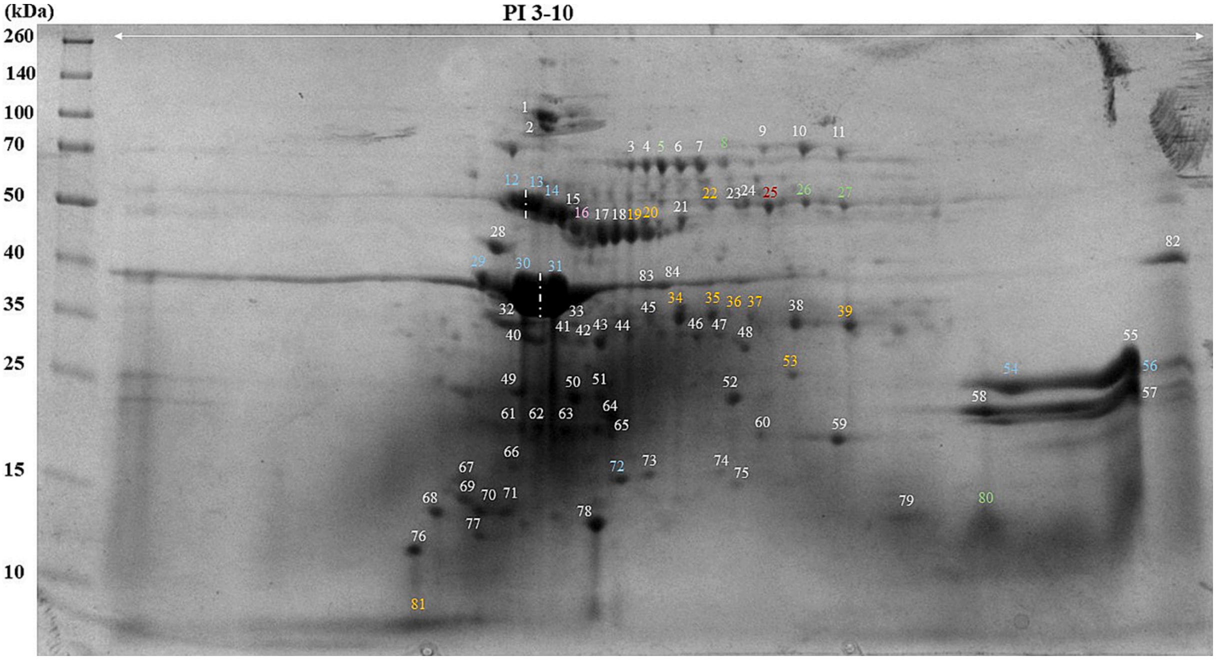

Fig. 2. 2D gel of salt-soluble proteins from cultivar Fuego under reducing condition and visualized with Coomassie Blue. Numbers refer to protein-spot numbers in Table 2. Blue, yellow, green and purple colors represent legumin subunits, vicilin and convicilin, putative sucrose binding protein, respectively. Red numbers represent multiple proteins identified. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The salt-soluble proteins from cultivar Fuego identified by MALDI-TOF from reducing 2D gel*

Protein spot	Accession No.	Protein name	m/z	Match	Score
5	B0BCL8	Convicilin	57,581	10	71
8	B0BCL8	Convicilin	57,581	11	94
25	B0BCL8	Convicilin	57,581	11	45
26	B0BCL8	Convicilin	57,581	17	151
27	B0BCL8	Convicilin	57,581	9	60
80	B0BCL8	Convicilin	57,581	9	53
19	I0B569	Vicilin	52,719	14	130
20	I0B569	Vicilin	52,719	12	118
25	I0B569	Vicilin	52,719	9	77
22	I0B569	Vicilin	52,719	8	81
34	I0B569	Vicilin	52,719	13	116
35	I0B569	Vicilin	52,719	7	58
36	I0B569	Vicilin	52,719	5	44
37	I0B569	Vicilin	52,719	9	68
39	I0B569	Vicilin	52,719	9	108
63	I0B569	Vicilin	52,719	9	56
81	I0B569	Vicilin	52,719	11	94
56	LEGB6/2/7	Legumin type B	37,089	7	43
72	Q03971	N-terminal incomplete legumin A1 pre-pro-polypeptide	57,043	7	66
31	Q03971	N-terminal incomplete legumin A1 pre-pro-polypeptide	57,043	6	56
12	Q43673	Legumin; legumin-related high molecular weight polypeptide	64,805	7	48
14	Q43673	Legumin; legumin-related high molecular weight polypeptide	64,805	3	40
13	Q43673	Legumin; legumin-related high molecular weight polypeptide	64,805	6	61
29	Q99304	Legumin A2 primary translation product	57,044	8	58
30	Q99304	Legumin A2 primary translation product	57,044	6	45
31	Q99304	Legumin A2 primary translation product	57,044	7	68
54	Q99304	Legumin A2 primary translation product	57,044	9	60
16	Q9AVP7	Putative sucrose binding protein	54,979	15	181

* The listed proteins are identified with significant hits (Score > Threshold score, 40).

represent legumin-related polypeptides. This result differed from previous 1D gel results, where bands at 48 kDa were regarded as vicilin (Tucci et al., 1991). Spots 54 and 56, having identical MW of 25 kDa and pI around 9, were identified as legumin-related polypeptide and legumin type B, respectively. These proteins are non-methionine containing legumin subunits, which could represent the basic β chain of legumin. Spot 72 with MW 15 kDa and pI 5.8 was identified as legumin A1 polypeptide, indicating the undergoing PTMs on polypeptides before they transferred to legumin (Tucci et al., 1991).

Compared to legumin, vicilin was detected with a wider distribution on the 2D gel. Eleven spots were identified as vicilin, among which spot 25 was annotated to both vicilin and convicilin. This observed co-location could be due to protein heterogeneity as a result of genetic variant and/or PTM heterogeneities (Tucci et al., 1991; Warsame et al., 2020). Spots 5 and 8 with MW of 70 kDa and pI around 6.5, and spots 26 and 27 located at MW of 57 kDa and pI around 7, were all identified as convicilin. Moreover, spot 80 with MW of 13 kDa and pI 8.2 and spot 81 with MW of 8 kDa and pI 4.5 were identified as convicilin and vicilin, respectively, instead of albumins as expected. This is likely due to the proteolytic products of vicilin and convicilin, as observed in earlier studies (Casey, Domoney, & Ellis, 1986; Gatehouse et al., 1981; Scholz et al., 1983). Notably, albumins were not identified in this analysis, which may result from the limited sensitivity of MALDI-TOF MS/MS, and the low abundance of albumins in faba beans. Collectively, these results confirm that globulins are the predominant protein components of faba bean salt-soluble extracts, and further that these globulins exhibit complex heterogeneities.

3.2.3. *Proteome analysis by nano LC-MS/MS*

To further quantify and identify differences in major and minor protein compositions across the different faba bean cultivars, the salt-extractable proteins were subjected to sensitive nano LC-MS/MS timsTOF II proteomics. Across the 10 cultivars, there were, in total, 6174 peptides matched to 103 unique proteins by SPIDER analysis, allowing point mutations. The identified proteins could be divided into seed storage proteins (26), enzymes (38), defense related proteins (11), different types of binding proteins (13), ANFs (8), transport proteins (5) and signal recognition proteins (2) according to biological functions (Table 3). Proteins from this analysis were searched against the *Vicia* protein library. The genus *Vicia* belongs to the Fabaceae family, comprising 180–210 species, among which *Vicia faba* is one of the most widespread legumes (Hanelt & Mettin, 1989). Thus, the *Vicia* protein library contains several entries for the same protein, where the proteins can be found in different *Vicia* genera, differing in AA sequence and MW. For instance, Table 3 shows, that vicilin is identified from both *Vicia faba* and *Vicia narbonensis*. Both proteins have the same AA length of 463, but, in *Vicia faba,* vicilin has a higher MW (53 kDa) compared to *Vicia narbonensis* vicilin (52 kDa), due to AA sequence variation among the two species. In the group of storage proteins, vicilins, convicilins and legumins from different species were identified. This can result from point mutations leading to the variations in peptide sequences annotated to the same proteins, but from different species.

Fig. 3 displays the abundance of the top 10 abundant proteins across the 10 different cultivars, hereafter denoted as “abundant proteins”. Overall, a total of 13 abundant proteins were collected across 10 cultivars due to the great overlap of the abundant proteins across cultivars. These abundant proteins included two types of vicilin, two types of convicilin, and four different legumin subunits, as well as α-1,4 glucan phosphorylase L isozyme (chloroplastic/amyloplastic), defensin-like protein, lipoxygenase, and putative albumin 1. As shown in Fig. 3a, in most cultivars, apart from Tiffany and Gl. Arabella, vicilin assigned to *Vicia faba* displayed higher abundance than vicilin assigned to *Vicia narbonensis*, where the abundance of two types of vicilin was at comparable levels. For identified vicilin from two species, the highest abundance was found in cultivar Stella, followed by Granit, and then Daisy. All cultivars showed a decreased trend from convicilin assigned to *Vicia faba* to convicilin assigned to *Vicia narbonensis* (Fig. 3b). The similar pattern observed could indicate that there is some similarity in protein polymorphisms across cultivars. The mutation implied by proteins assigned to other *Vicia species* accounts for a small proportion, compared to normal proteins assigned to *Vicia faba* in most cultivars. Apollo had the highest convicilin (*Vicia faba*), but overall, most of the cultivars displayed a similar pattern, except Fuego and Lynx, which had a relatively lower abundance of convicilin (*Vicia faba*). The abundances of both convicilins were similar in Daisy and Tiffany. The identified two vicilin variants from these two species shared a similar AA sequence, which was also observed in convicilin, demonstrating the heterogeneity of faba bean 7S globulins. This can result from PTMs and mutations in the AA sequence, which were monitored through the SIPDER analysis. In SPIDER analysis, the abundance of a protein is an expression of the sum of the peak area of all peptides annotated to this protein. Thus, larger proteins tend to get higher abundance compared to smaller proteins. Four abundant legumin subunits were detected, demonstrating the complexity of faba bean globulins (Fig. 3c). Cultivars Tiffany and Birgit showed the highest abundance in legumin-related high molecular weight polypeptide (*Vicia faba* var. *minor*) and legumin type B (*Vicia faba*), respectively. Apollo had the highest abundance of both legumin

Table 3
Identified proteins by SPIDER analysis of faba bean proteins using Uniprot *Vicia* protein library.

Function	Protein ID	Accession	Description	Organism
Storage protein	13,256	I0B569	Vicilin	*Vicia faba*
	13,268	Q41677	Vicilin	*Vicia narbonensis*
	13,351	Q41674	Convicilin	*Vicia narbonensis*
	13,260	B0BCL8	Convicilin	*Vicia faba*
	13,279	B0BCL7	Convicilin	*Vicia faba*
	13,337	B0BCK9	Cvc protein	*Vicia disperma*
	13,349	B0BCL0	Cvc protein	*Vicia bithynica*
	13,350	B0BCL1	Cvc protein	*Vicia villosa*
	13,363	B0BCL3	Convicilin	*Vicia lutea*
	13,364	B0BCL2	Convicilin	*Vicia peregrina*
	13,373	B0BCL4	Convicilin	*Vicia articulata*
	13,397	B0BCL5	Convicilin	*Vicia ervilia*
	13,259	Q43673	Legumin; legumin-related high molecular weight polypeptide	*Vicia faba var. minor*
	13,501	Q43672	Legumin; legumin-related high molecular weight polypeptide	*Vicia faba var. minor*
	13,523	Q43674	Legumin; legumin-related high molecular weight polypeptide	*Vicia faba var. minor*
	13,264	Q99304	Legumin A2 primary translation product	*Vicia faba var. minor*
	13,271	Q41676	Legumin A	*Vicia narbonensis*
	13,273	Q41702	Legumin A	*Vicia sativa*
	13,258	P05190	Legumin type B	*Vicia faba*
	13,282	Q41703	Legumin B	*Vicia sativa*
	13,266	P16080	Legumin type B	*Vicia faba*
	13,267	P16079	Legumin type B	*Vicia faba*
	13,263	Q03971	N-terminal incomplete legumin A1 pre-pro-polypeptide	*Vicia faba var. minor*
	26,829	Q6A1C7	Putative albumin 1	*Vicia hirsuta*
	13,262	Q9AVP7	Putative sucrose binding protein	*Vicia faba var. minor*
	13,398	Q43671	Storage protein	*Vicia faba var. minor*
Enzyme	13,346	R4IV45	ATP synthase subunit alpha	*Vicia faba*
	13,394	Q7DLK9	Guanine nucleotide regulatory protein	*Vicia faba*
	13,436	Q41663	Nodulin homologous to narbonin	*Vicia faba*
	13,261	P53536	Alpha-1 4 glucan phosphorylase L isozyme chloroplastic/amyloplastic	*Vicia faba*
	13,274	P52416	Glucose-1-phosphate adenylyltransferase small subunit 1 chloroplastic	*Vicia faba*
	13,275	P52417	Glucose-1-phosphate adenylyltransferase small subunit 2 chloroplastic	*Vicia faba*
	13,276	P53537	Alpha-glucan phosphorylase H isozyme	*Vicia faba*
	13,280	O82723	Phosphoenolpyruvate carboxylase	*Vicia faba*
	13,281	A0A023I2J6	Ribulose bisphosphate carboxylase large chain	*Vicia faba*
	13,305	O82724	Pepc2 protein (Phosphoenolpyruvate carboxylase protein)	*Vicia faba*
	13,325	Q43876	Probable sucrose-phosphate synthase	*Vicia faba*
	13,372	O64456	Peptidyl-prolyl cis-trans isomerase	*Vicia faba*
	13,374	H9BPH8	Catalase	*Vicia faba*
	13,411	H9BPH7	Superoxide dismutase [Cu—Zn]	*Vicia faba*
	13,414	A0A1Q1N6Q3	Superoxide dismutase	*Vicia sativa*
	13,423	P31926	Sucrose synthase	*Vicia faba*
	13,427	B6RPT3	phosphogluconate dehydrogenase (NADP(+)-dependent decarboxylating)	*Vicia faba*
	13,455	Q41651	Peptidyl-prolyl cis-trans isomerase chloroplastic	*Vicia faba*
	13,461	O04919	Lipoxygenase	*Vicia faba*
	13,465	Q41671	Pre-pro-cysteine proteinase	*Vicia faba*
	13,481	Q41649	FK506-binding protein 2	*Vicia faba*
	13,483	O04287	Peptidyl-prolyl cis-trans isomerase FKBP12	*Vicia faba*
	13,509	A0A2I6PNB2	Endoglucanase	*Vicia faba*
	13,518	A2ICG3	OASTL	*Vicia sativa*
	13,550	D7RTA5	Glyceraldehyde-3-phosphate dehydrogenase	*Vicia sativa*
	13,561	A0A9E9GIS8	Delta-1-pyrroline-5-carboxylate synthase isoform X2	*Vicia sativa*
	13,565	A5JJV0	dihydroorotase	*Vicia faba*
	13,699	M1GCP8	Glutamate decarboxylase	*Vicia faba*
	13,725	O82102	Cysteine proteinase	*Vicia sativa*
	13,845	O78672	Glycerol-3-phosphateacyl transferase chloroplastic	*Vicia faba*
	14,298	Q7DLK8	Guanine nucleotide regulatory protein	*Vicia faba*
	14,560	Q0EAF2	Serine/threonine-protein phosphatase	*Vicia faba*
	15,168	S5RBC6	Mitogen activate protein kinase	*Vicia faba*
	15,189	D3KZ44	Ornithine aminotransferase	*Vicia villosa*
	15,325	O24545	Cysteine proteinase	*Vicia sativa*
	18,787	B5LRC1	Cytosolic copper/zinc superoxide dismutase	*Vicia faba*
	18,994	Q7M228	pyruvate decarboxylase	*Vicia faba*
	19,772	B5LRC5	Glutathione reductase	*Vicia faba*
Defense like protein	13,601	L7UP05	Heat shock protein 17.9	*Vicia faba*
	13,609	A0A1X7BYR4	Pathogenesis-related protein 10	*Vicia faba*
	13,365	A0A1X7QH12	chitinase	*Vicia faba*
	13,504	R4IKN8	ABA-17 responsive protein	*Vicia faba*
	13,548	B5TGN0	Defensin-like protein	*Vicia faba*
	13,549	B5TGN4	Defensin-like protein	*Vicia faba*
	13,681	C4TP26	Dehydrin a OS=*Vicia monantha*	
	15,742	B5TGN1	Defensin-like protein	*Vicia faba*
	18,794	P81456	Defensin-like protein 1	*Vicia faba*
	23,155	C4TP27	Dehydrin b OS=*Vicia monantha*	

(*continued on next page*)

Table 3 (*continued*)

Function	Protein ID	Accession	Description	Organism
	42,408	H9BIP0	Pathogenesis-related protein 5	*Vicia faba*
Binding	13,335	P42653	14–3-3-like protein A	*Vicia faba*
	13,343	P42654	14–3-3-like protein B	*Vicia faba*
	13,375	Q9FXL2	Vf14–3-3c protein	*Vicia faba*
	13,392	Q9FXL1	Vf14–3-3d protein	*Vicia faba*
	13,594	O04237	Transcription factor	*Vicia faba var. minor*
	13,456	Q84NG0	Histone H1	*Vicia faba*
	14,805	P40620	HMG1/2-like protein	*Vicia faba*
	15,095	L7V1D2	Histone H1 subtype 6	*Vicia unijuga*
	13,462	B5TGN5	Metallothionein-like protein	*Vicia faba*
	13,338	A0A059PAV5	Actin	*Vicia faba*
	13,366	D5LN06	Actin	*Vicia sativa*
	13,390	D5M8H2	Actin	*Vicia sativa*
	13,400	C7F8N1	Actin	*Vicia faba*
ANF	13,497	P24661	Bowman-Birk type proteinase inhibitor	*Vicia faba*
	13,508	P02871	Favin	*Vicia faba*
	13,587	Q8RVW8	Lectin	*Vicia faba*
	13,608	P08820	Subtilisin inhibitor	*Vicia faba*
	13,791	B5TGM9	Fabatin	*Vicia faba*
	14,664	P01065	Bowman-Birk type proteinase inhibitor	*Vicia sativa* subsp. *nigra*
	17,756	P02868	Mannose/glucose-specific lectin alpha chain	*Vicia cracca*
	19,007	Q9ZRM4	Lectin	*Vicia cracca*
Transport	13,597	P00288	Plastocyanin	*Vicia faba*
	14,364	A0A0Y0UTM1	ATP synthase subunit beta chloroplastic	*Vicia villosa*
	13,440	A0A173H230	Early nodulin-like protein 2	*Vicia faba*
	13,342	P38548	GTP-binding nuclear protein Ran/TC4	*Vicia faba*
	13,270	O24534	Elongation factor 1-alpha	*Vicia faba*
Recognition signals	13,522	Q9ZRW9	Polyubiquitin	*Vicia faba*
	18,802	Q9M3N3	Putative kinetochore protein	*Vicia faba var. minor*

A2 primary translation product (*Vicia faba*) and legumin A2 (*Vicia sativa*). Generally, Fuego and Gl. Arabella showed lower abundances for all the legumins among the 10 cultivars. Granit had the highest amount of putative sucrose binding protein (*Vicia faba* var. *minor*), followed by Apollo and Tiffany (Fig. 3d). The abundance of defense-like protein (*Vicia faba*) and lipoxygenase (*Vicia faba*) was comparable across cultivars (Fig. 3D), whereas the abundance of α-1,4 glucan phosphorylase L isozyme (chloroplastic/amyloplastic) and putative albumin 1 displayed apparent difference across cultivars. Collectively, each of the abundant proteins showed distinct abundances across cultivars, but similar trends between different proteins were also observed in some cultivars. These results together revealed the interacting complexity of faba bean protein polymorphism in terms of genotypes.

By applying label-free quantification based on the top 3 unique peptides per identified protein, 61 unique proteins were quantified. The relative abundance of the quantified proteins is shown in Fig. 4. 27 proteins were quantified with higher than 1 % relative abundance across the 10 cultivars, displaying significant differences in protein composition. In combination, the storage proteins composed of globulins accounted for 68–82 % of the total quantified protein. This result was also reflected in 1D and 2D gels, where intense legumin and vicilin bands/spots were observed. As a vital source of dietary protein, the storage protein in legumes which in general accounts for 50–90 % of total seed proteins is important and the contrast cereals, where the storage proteins generally account for less than 50 % of the proteins (Nikolić et al., 2012; Shewry & Halford, 2002). Among globulins, vicilin accounting 23–56 % of total globulins appeared to be the dominant protein in all cultivars, this was followed by legumin (18–51 %) and convicilin (15–36 %). Putative sucrose binding protein was a membrane-associated protein accounting for 0–9 % across cultivars, which was also identified by MALDI-TOF. The sucrose binding protein plays a key role in transporting sucrose during the developing faba bean seeds (Farley & Patrick, 2000). Research also found that putative sucrose binding protein exhibits GTP binding activity as a membrane-associated protein in soybean and mung bean (Pirovani et al., 2002; Wang et al., 2009).

The abundance and composition of faba bean globulins varied across cultivars (Fig. 5) illustrating that the protein compositions were affected by genotypes. This difference between cultivars could not be revealed by using 1D gels. The 7S globulins vicilin and convicilin showed higher abundance than 11 S globulin legumin, except in cultivar Granit, where a balanced ratio 1:1 was detected. Fuego had the lowest ratio of L/V, whereas legumin was 4 times less abundant than vicilin and convicilin. The L/V ratio between Apollo and Daisy, and Gl. Arabella and Birgit were comparable. These L/V ratios were different from previous studies on faba bean globulin compositions, where legumin was found to be 1 to 3 times more abundant than vicilin and convicilin (Singhal et al., 2016; Warsame et al., 2020). Warsame et al. (2020) used size exclusion HPLC to separate faba bean legumin from vicilin and convicilin to calculate the ratio of L/V. This method resulted in limited separation of legumin and vicilin as peaks of legumin and vicilin were co-eluted. Singhal et al. (2016) applied 1D SDS-PAGE to quantify the ratio of L/V without considering the overlaps of different protein subunits in protein bands. Thus, the difference in applied methods largely affects the value of L/V. Moreover, the L/V ratio was also manipulated by genotypes and growing conditions because vicilin formation precedes legumin, which is synthesized during the later stages of seed development (Wright & Boulter, 1972). Therefore, the temperature and water supply during maturation can even provoke an inversion of L/V (Manteuffel et al., 1976). In addition, the correlation between L/V ratio and protein content, S-AA content in faba bean seeds was far from conclusion.

Apart from storage proteins, the most abundant protein was found to be defense-like proteins (2–8 %), followed by the ANF proteins BBI (3–7 %), and lectin (1–4 %) (Table S1). The function of the defense-related proteins in plants is to resist a variety of stresses during growth, such as pathogen invasion (Souza et al., 2017). In this study, the quantified defense-like protein plays a role for the plant in response to fungus. The BBI and lectins are synthesized in response to attacking pests or pathogens in legumes (Ryan, 1990). They are the main ANF proteins affecting protein digestion and mineral absorption. For human consumption, the enrichment of BBI and lectins in faba bean protein isolates and concentrates may hinder faba bean utilization, and the elimination of these ANFs in faba beans through e.g. plant breeding is still in progress. The relatively high content of lipoxygenase is also evident from the 1D gel. The presence of this enzyme may be related to off-flavor formation in faba bean and specifically in the generation of volatiles related to the

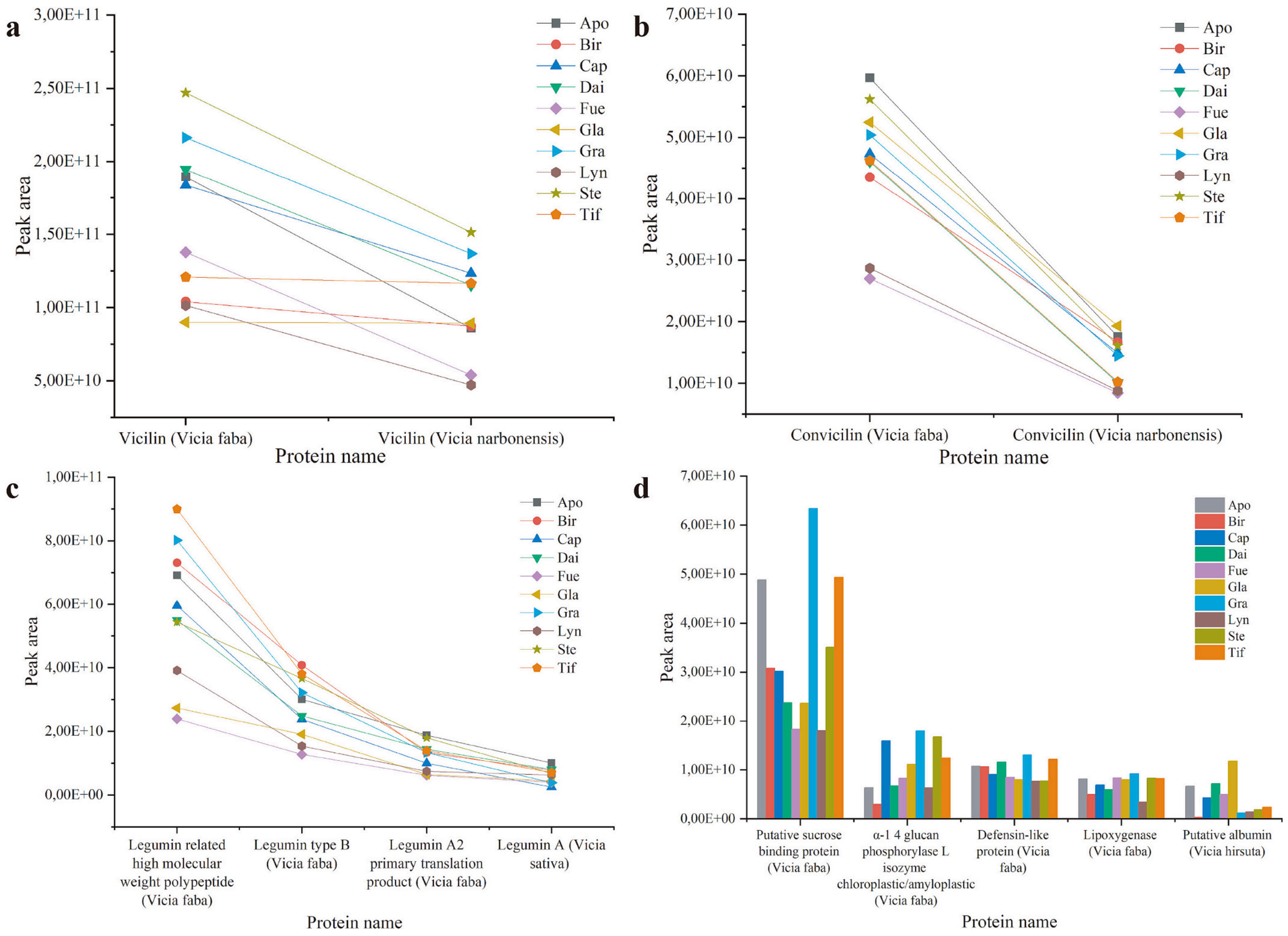

Fig. 3. Peak area of top 10 abundant proteins in 10 different cultivars. Peak area of a. vicilins, b. convicilins, c. legumin subunits, d. non-globulins in 10 different faba bean cultivars. Lines in parallel reflect the same variation trend of proteins across cultivars. Otherwise, it reflects a different trend of proteins across cultivars.

beany flavor of faba bean (Jiang et al., 2016). Moreover, there are more quantified bio-functional proteins accounting for around 1 % of total proteins which could not be identified by the 1D and 2D gels, showing the high sensitivity and precision of the tims TOF Pro II.

3.3. AA composition

Due to the limitation of protein extraction, the total AA composition of 10 cultivars of faba beans was determined using acid hydrolysis and LC-MS/MS (Table 1). In total, around 850 mg AAs, including 400 mg essential amino acids (EAAs), were detected per gram protein. This result was in line with other studies showing that nearly 50 % of faba bean protein is composed of nonessential AAs (e.g. glutamic acid, aspartic acid, arginine, serine) (Warsame et al., 2018). The highest content of EAAs was histidine (64–82 mg/g protein), followed by leucine (52–64 mg/g protein) and lysine (49–57 mg/g protein). Histidine, together with its metabolites, has multiple benefits on human health such as glucoregulatory and cognitive function outcomes (Thalacker-Mercer & Gheller, 2020). The histidine content was two times higher in this study compared to previous studies on faba bean AAs, as a reflection of genotype difference (Makkar et al., 1997; Grela et al., 2017). The biosynthesis of histidine involves 11 reactions that are integrated with various metabolic pathways, including those of pyrimidines, folates, and tryptophan (Stepansky & Leustek, 2006). Moreover, the amino acids composition in faba bean seeds can be modulated by genotypes and growing conditions e.g. salt stress during seed development (Ghassemi-Golezani & Samea-Andabjadid, 2022; Migdadi et al., 2016), although the underlying mechanisms remain unclear. Among the 10 cultivars, the highest EAA content was detected in the cultivar Stella and the lowest content in the cultivar Daisy. The cultivars Birgit and Stella showed the highest amount of histidine and methionine, respectively, while the cultivars Fuego and Tiffany showed the lowest contents. Despite the AA composition varied across cultivars, no statistical significance was observed. These results demonstrate the similarity of AA sequences of faba bean proteins across cultivars.

The concentration of S-AA methionine and cysteine were the lowest in all cultivars, with cultivar Tiffany showing the lowest values (1.63 and 0.93 mg/g protein, respectively). Faba beans are broadly deficient in S-AA due to limited numbers of S-AA in storage proteins as legumin A and convicilin (Makkar et al., 1997; Liu et al., 2017, Table S2). Another source of S-AA is trypsin inhibitors (e.g. BBI) even though they account for a small proportion of faba bean seed proteins. However, cultivars Stella and Birgit had the highest methionine and cysteine concentrations in contrast to Apollo, which contained the most abundant convicilin and legumin A subunits, indicating that no positive correlations were evident between the concentration of S-AA and S-AA containing proteins. This may partly be related to free AAs in faba bean seeds and/or proteins that were not extracted for the proteomic analysis. Moreover, gamma-amino-n-butyric acid, L-ornithine and taurine with healthy benefits for humans were detected in several cultivars of faba beans. Of note, taurine is one of the sulfur sources in faba beans. The S-AA contents in faba bean can increase by applying sulfur and potassium

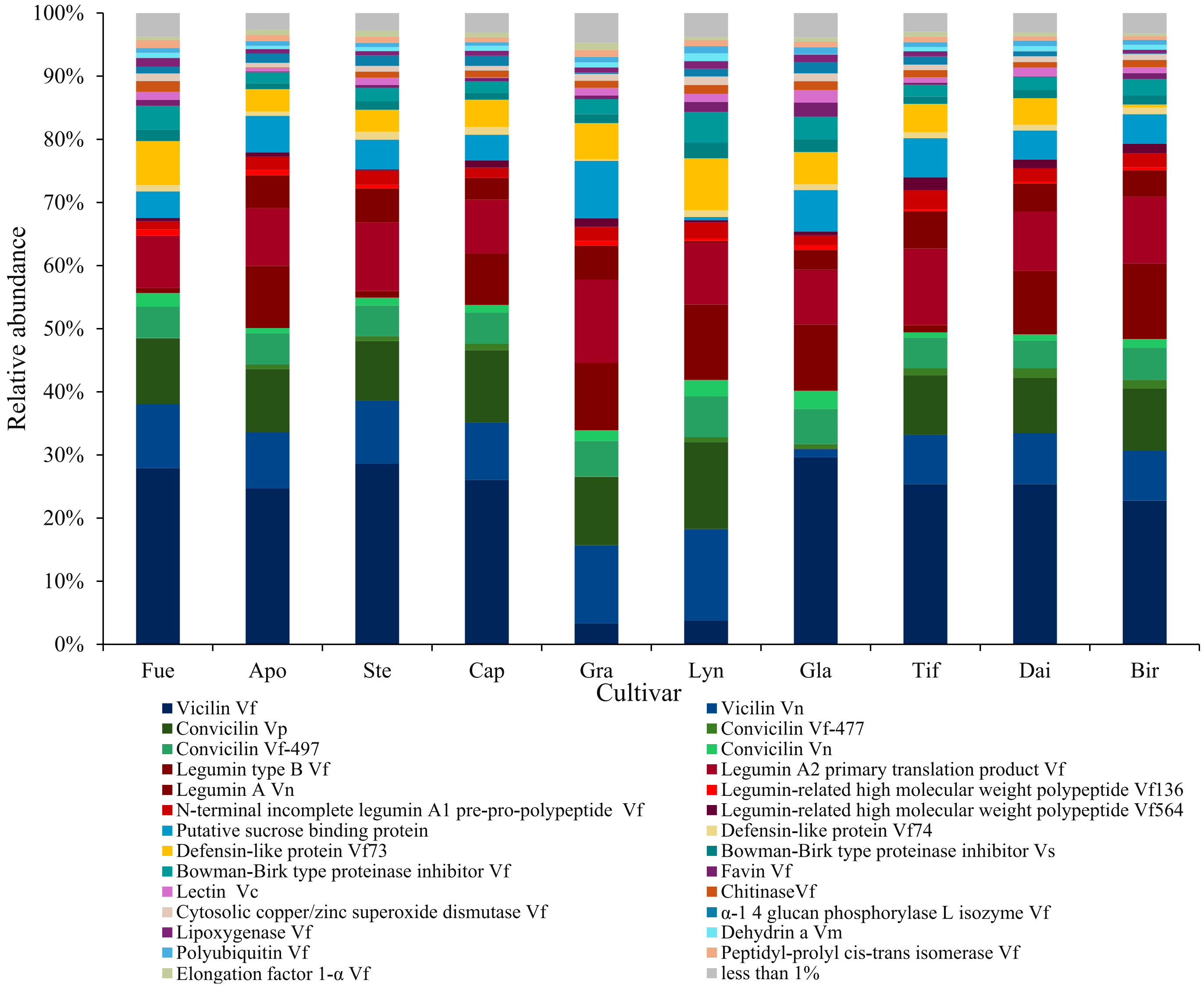

Fig. 4. The relative abundance of faba bean salt-soluble proteins. The total quantified proteins were regarded as 100 %. From the bottom, the colour blue showed the relative abundance of different vicilin, green showed the relative abundance of convicilin, and red showed the relative abundance of legumin subunits. The grey colour on the top represented the sum of proteins with relative abundance less than 1 % of total proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

simultaneously (Barłóg et al., 2019). The concentration of phenylalanine, threonine, and lysine was comparable to previous studies on faba bean AA (Millar et al., 2019).

4. Conclusions

In this study, the 1D gel displayed that the predominant protein bands were consistent across all faba bean cultivars. The 2D gel combined with MALDI TOF showed overall faba bean salt-soluble protein composition in faba beans, including legumin subunits, vicilin and convicilin, followed by putative sucrose binding protein. The protein composition and abundance of faba bean salt-soluble proteins were further detected by nano LC-MS/MS timsTOF Pro II proteomic analysis. As expected, the results demonstrated that legumin, vicilin and convicilin are the predominant proteins in faba bean seeds. In addition, proteins with diverse functions, such as the proteins, enzymes, and albumins, including proteinase inhibitors and lectins, were quantified and showed great variations across cultivars. The ratio of L/V also varied across cultivars, with 7S globulins vicilin and convicilin showing higher abundance than 11 S globulin legumin. For AA composition, all the faba bean cultivars displayed high levels of histidine and low levels of S-AA. The AA concentrations were different across cultivars, among which cultivar Stella showed the highest EAA content. This study applied the high accuracy and sensitivity of nano LC-MS/MS on salt-soluble faba bean proteins to screen the protein profiles and disclose their variations across cultivars. It revealed the prevalence of storage proteins but clear variabilities in protein abundances among different cultivars, which enable future studies to breed cultivars with desired protein compositions. Moreover, quantified minor proteins like enzymes can be targeted and modulated to generate desired phenotypes, and future studies can investigate the digestibility of faba beans with different protein compositions to better understand the protein quality and nutritional value across different cultivars.

CRediT authorship contribution statement

Tianzhen Xiao: Writing – original draft, Visualization, Validation, Software, Funding acquisition, Formal analysis. **Ida Schwartz Roland:** Writing – review & editing, Methodology, Data curation. **Søren Drud-Heydary Nielsen:** Software, Methodology. **Thao T. Le:** Writing –

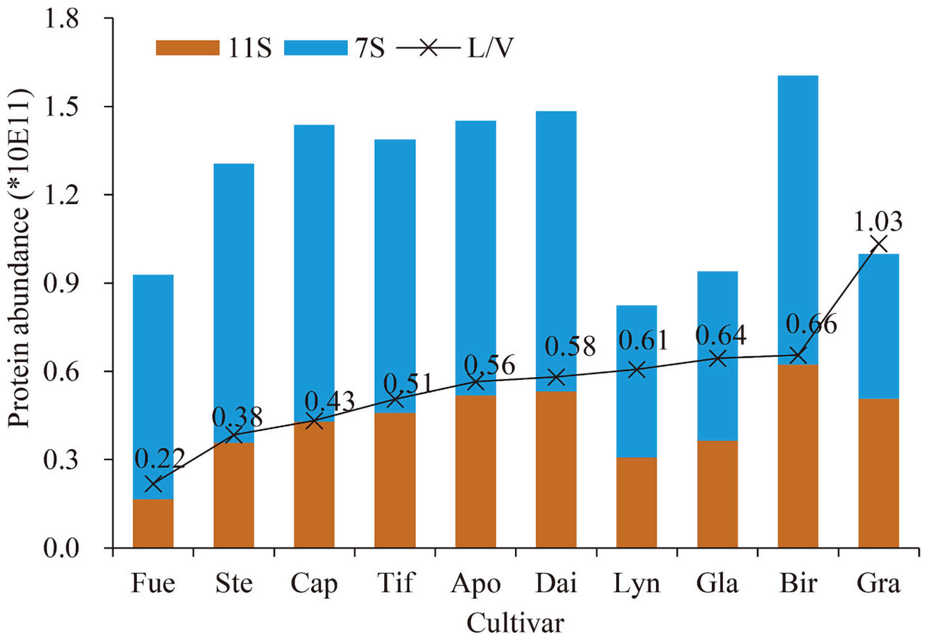

Fig. 5. The 11S legumin/7 s vicilin and convicilin ratio (L/V) in 10 cultivars.

review & editing, Methodology, Data curation. **Esben Olesen:** Writing – review & editing, Software, Investigation, Data curation. **Lotte Bach Larsen:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis. **Nina Aagaard Poulsen:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis.

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.

Acknowledgement

We express our gratitude to the suppliers of faba beans for providing the materials essential for this study. This research was part of the project ØkoFaba financed by the Danish Organic Farming Levy Foundation and the China Scholarship Council, China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.144225.

Data availability

Data will be made available on request.

References

AACC. (2025). Approved Methods of Analysis, 11th Ed. Method 46–30.01, Crude Protein - Combustion Method, St. Paul, MN. *USA: American Association of Cereal Chemists*.

Asao, T., Imai, F., Tsuji, I., Tashiro, M., Iwami, K., & Ibuki, F. (1991). The amino acid sequence of a Bowman-Birk type proteinase inhibitor from faba beans (*Vicia faba* L.). *The Journal of Biochemistry, 110*(6), 951–955.

Barłóg, P., Grzebisz, W., & Łukowiak, R. (2019). The effect of potassium and sulfur fertilization on seed quality of faba bean (*Vicia faba* L.). *Agronomy, 9*(4), 209.

Casey, R., Domoney, C., & Ellis, N. (1986). Legume storage proteins and their genes. *Oxford Surveys of Plant Molecular and Cell Biology, 3*, 1–95.

Chelius, D., & Bondarenko, P. V. (2002). Quantitative profiling of proteins in complex mixtures using liquid chromatography and mass spectrometry. *Journal of Proteome Research, 1*(4), 317–323.

Croy, R. R., Gatehouse, J. A., Evans, I. M., & Boulter, D. (1980). Characterisation of the storage protein subunits synthesized in vitro by polyribosomes and RNA from developing pea (*Pisum sativum* L.) I. *Legumin. Planta, 148*, 49–56.

Diep, T. T., Pook, C., & Yoo, M. J. Y. (2020). Physicochemical properties and proximate composition of tamarillo (*Solanum betaceum* Cav.) fruits from New Zealand. *Journal of Food Composition and Analysis, 92*, 103,563.

El Fiel, H. E. A., El Tinay, A. H., & Elsheikh, E. A. (2002). Effect of nutritional status of faba bean (*Vicia faba* L.) on protein solubility profiles. *Food Chemistry, 76*(2), 219–223.

Farley, S. J., Patrick, J. W., & Offler, C. E. (2000). Functional transfer cells differentiate in cultured cotyledons of *Vicia faba* L. seeds. *Protoplasma, 214*, 102–117.

Fuchs, J., & Schubert, I. (1995). Localization of seed protein genes on metaphase chromosomes of *Vicia faba* via fluorescence in situ hybridization. *Chromosome Research, 3*, 94–100.

Gatehouse, J. A., Croy, R. R., Morton, H., Tyler, M., & Boulter, D. (1981). Characterisation and subunit structures of the vicilin storage proteins of pea (*Pisum sativum* L.). *European Journal of Biochemistry, 118*(3), 627–633.

Geil, P. B., & Anderson, J. W. (1994). Nutrition and health implications of dry beans: a review. *Journal of the American College of Nutrition, 13*(6), 549–558.

Ghassemi-Golezani, K., & Samea-Andabjadid, S. (2022). Exogenous cytokinin and salicylic acid improve amino acid content and composition of faba bean seeds under salt stress. *Gesunde Pflanzen, 74*(4), 935–945.

Grela, E. R., Kiczorowska, B., Samolińska, W., Matras, J., Kiczorowski, P., Rybiński, W., & Hanczakowska, E. (2017). Chemical composition of leguminous seeds: part I—content of basic nutrients, amino acids, phytochemical compounds, and antioxidant activity. *European Food Research and Technology, 243*, 1385–1395.

Griffiths, D. W., & Lawes, D. A. (1978). Variation in the crude protein content of field beans (*Vicia faba* L.) in relation to the possible improvement of the protein content of the crop. *Euphytica, 27*(2), 487–495.

Gueguen, J., & Cerletti, P. (1994). Proteins of some legume seeds: soybean, pea, fababean and lupin. In *New and developing sources of food proteins* (pp. 145–193). Boston, MA: Springer US.

Hanelt, P., & Mettin, D. (1989). Biosystematics of the genus Vicia L.(Leguminosae). *Annual Review of Ecology and Systematics*, 199–223.

Husband, H., Ferreira, S., Bu, F., Feyzi, S., & Ismail, B. P. (2024). Pea protein globulins: Does their relative ratio matter? *Food Hydrocolloids, 148*(109), 429.

Iqbal, A., Khalil, I. A., Ateeq, N., & Khan, M. S. (2006). Nutritional quality of important food legumes. *Food Chemistry, 97*(2), 331–335.

Jensen, E. S., Peoples, M. B., & Hauggaard-Nielsen, H. (2010). Faba bean in cropping systems. *Field Crops Research, 115*(3), 203–216.

Jensen, H. B., Poulsen, N. A., Møller, H. S., Stensballe, A., & Larsen, L. B. (2012). Comparative proteomic analysis of casein and whey as prepared by chymosin-induced separation, isoelectric precipitation or ultracentrifugation. *Journal of Dairy Research, 79*(4), 451–458.

Jiang, Z. Q., Pulkkinen, M., Wang, Y. J., Lampi, A. M., Stoddard, F. L., Salovaara, H., & Sontag-Strohm, T. (2016). Faba bean flavor and technological property improvement by thermal pre-treatments. *LWT- Food Science and Technology, 68*, 295–305.

Jiménez-Munoz, L., Nielsen, S. D. H., & Corredig, M. (2024). Changes in potato peptide bioactivity after gastrointestinal digestion. An in silico and in vitro study of different patatin-rich potato protein isolate matrices. Food Chemistry. *Advances, 4*, 100,679.

Kimura, A., Fukuda, T., Zhang, M., Motoyama, S., Maruyama, N., & Utsumi, S. (2008). Comparison of Physicochemical Properties of 7S and 11S Globulins from Pea, Fava Bean, Cowpea, and French Bean with Those of Soybean French Bean 7S Globulin Exhibits Excellent Properties. *Journal of Agricultural and Food Chemistry, 56*(21), 10,273–10,279.

Knudsen, L. J., Rauh, V., Pedersen, J. N., Dekker, P., Otzen, D. E., Larsen, L. B., & Nielsen, S. D. (2024). Insight into protein crosslinking and casein polymerization in pre-and posthydrolyzed lactose-free ultra-high-temperature milk during long-term storage. *Journal of Dairy Science, 107*(12), 10497–10511.

Koivunen, E., Partanen, K., Perttilä, S., Palander, S., Tuunainen, P., & Valaja, J. (2016). Digestibility and energy value of pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.) and blue lupin (narrow-leaf) (*Lupinus angustifolius*) seeds in broilers. *Animal Feed Science and Technology, 218*, 120–127.

Labba, I. C. M., Frøkiær, H., & Sandberg, A. S. (2021). Nutritional and antinutritional composition of fava bean (*Vicia faba* L., var. minor) cultivars. *Food Research International, 140*, 110,038.

Liener, I. E. (1994). Implications of antinutritional components in soybean foods. *Critical Reviews in Food Science and Nutrition, 34*(1), 31–67.

Liu, Y., Wu, X., Hou, W., Li, P., Sha, W., & Tian, Y. (2017). Structure and function of seed storage proteins in faba bean (*Vicia faba* L.). 3. *Biotech, 7*, 1–14.

Makkar, H. P., Becker, K., Abel, H. J., & Pawelzik, E. (1997). Nutrient contents, rumen protein degradability and antinutritional factors in some colour-and white-flowering cultivars of *Vicia faba* beans. *Journal of the Science of Food and Agriculture, 75*(4), 511–520.

Manteuffel, R., Müntz, K., Püchel, M., & Scholz, G. (1976). Phase-dependent changes of DNA, RNA and protein accumulation during the ontogenesis of broad bean seeds (*Vicia faba* L., var. minor). *Biochemie und Physiologie der Pflanzen, 169*(6), 595–605.

Mertens, C., Dehon, L., Bourgeois, A., Verhaeghe-Cartrysse, C., & Blecker, C. (2012). Agronomical factors influencing the legumin/vicilin ratio in pea (*Pisum sativum* L.) seeds. *Journal of the Science of Food and Agriculture, 92*(8), 1591–1596.

Migdadi, H. M., El-Harty, E. H., Salamh, A., & Khan, M. A. (2016). Yield and proline content of faba bean genotypes under water stress treatments. *JAPS: Journal of Animal & Plant Sciences, 26*(6).

Millar, K. A., Gallagher, E., Burke, R., McCarthy, S., & Barry-Ryan, C. (2019). Proximate composition and anti-nutritional factors of fava-bean (*Vicia faba*), green-pea and yellow-pea (*Pisum sativum*) flour. *Journal of Food Composition and Analysis, 82*(103), 233.

Mosse, J. (1990). Nitrogen-to-protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content. *Journal of Agricultural and Food Chemistry, 38*(1), 18–24.

Multari, S., Stewart, D., & Russell, W. R. (2015). Potential of fava bean as future protein supply to partially replace meat intake in the human diet. *Comprehensive Reviews in Food Science and Food Safety, 14*(5), 511–522.

Müntz, K., Horstmann, C., & Schlesier, B. (1999). Vicia globulins. In P. R. Shewry, & R. Casey (Eds.), *Seed Proteins* (pp. 259–284). Dordrecht: Springer.

Mustafa, A., Åman, P., Andersson, R., & Kamal-Eldin, A. (2007). Analysis of free amino acids in cereal products. *Food Chemistry, 105*(1), 317–324.

Nikolić, Z., Đorđević, V., Torbica, A., & Mikić, A. (2012). Legumes seed storage proteins characterisation by SDS-PAGE and Lab-on-a-Chip electrophoresis. *Journal of Food Composition and Analysis, 28*(2), 75–80.

O'Kane, F. E., Happe, R. P., Vereijken, J. M., Gruppen, H., & van Boekel, M. A. (2004). Characterisation of pea vicilin. 1. Denoting convicilin as the α-subunit of the Pisum vicilin family. *Journal of Agricultural and Food Chemistry, 52*(10), 3141–3148.

Park, S. J., Kim, T. W., & Baik, B. K. (2010). Relationship between proportion and composition of albumins, and in vitro protein digestibility of raw and cooked pea seeds (*Pisum sativum* L.). *Journal of the Science of Food and Agriculture, 90*(10), 1719–1725.

Pirovani, C. P., Macêdo, J. N. A., Contim, L. A. S., Matrangolo, F. S., Loureiro, M. E., & Fontes, E. P. (2002). A sucrose binding protein homologue from soybean exhibits GTP-binding activity that functions independently of sucrose transport activity. *European Journal of Biochemistry, 269*(16), 3998–4008.

Pulkkinen, M., Zhou, X., Lampi, A. M., & Piironen, V. (2016). Determination and stability of divicine and isouramil produced by enzymatic hydrolysis of vicine and convicine of faba bean. *Food Chemistry, 212*, 10–19.

Reeke, G. N., Jr., & Becker, J. W. (1986). Three-dimensional structure of favin: saccharide binding-cyclic permutation in leguminous lectins. *Science, 234*(4780), 1108–1111.

Roland, I. S., Le, T. T., Chen, T., Aguilera-Toro, M., Nielsen, S. D.-H., Larsen, L. B., & Poulsen, N. A. (2024). Storage stability of plant-based drinks related to proteolysis and generation of free amino acids. *Foods, 13*, 367.

Ryan, C. A. (1990). *Protease inhibitors in plants: genes for improving defences against insects and pathogens*.

Sáenz de Miera, L. E., Ramos, J., & Pérez De La Vega, M. (2008). A comparative study of convicilin storage protein gene sequences in species of the tribe Vicieae. *Genome, 51* (7), 511–523.

Salazar, C., Armenta, J. M., & Shulaev, V. (2012). An UPLC-ESI-MS/MS assay using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization for targeted amino acid analysis: application to screening of *Arabidopsis thaliana* mutants. *Metabolites, 2* (3), 398–428.

Scholz, G., Manteufell, R., Muntz, K., & Rudolph, A. (1983). Low-Molecular-Weight Polypeptides of Vicilin from *Vicia faba* L. are Product of Proteolytic Breakdown. *European Journal of Biochemistry, 132*(1), 103–107.

Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany, 53*(370), 947–958.

Shewry, P. R., Napier, J. A., & Tatham, A. S. (1995). Seed storage proteins: structures and biosynthesis. *The Plant Cell, 7*(7), 945.

Shi, D., & Nickerson, M. T. (2022). Comparative evaluation of the functionality of faba bean protein isolates with major legume proteins in the market. *Cereal Chemistry, 99* (6), 1246–1260.

Singhal, A., Stone, A. K., Vandenberg, A., Tyler, R., & Nickerson, M. T. (2016). Effect of genotype on the physicochemical and functional attributes of faba bean (*Vicia faba* L.) protein isolates. *Food Science and Biotechnology, 25*, 1513–1522.

Souza, T. P., Dias, R. O., & Silva-Filho, M. C. (2017). Defense-related proteins involved in sugarcane responses to biotic stress. *Genetics and Molecular Biology, 40*, 360–372.

Stepansky, A., & Leustek, T. (2006). *Histidine biosynthesis in plants. Amino acids. 30* pp. 127–142).

Stoddard, F. L., Balko, C., Erskine, W., Khan, H. R., Link, W., & Sarker, A. (2006). Screening techniques and sources of resistance to abiotic stresses in cool-season food legumes. *Euphytica, 147*, 167–186.

Thalacker-Mercer, A. E., & Gheller, M. E. (2020). Benefits and adverse effects of histidine supplementation. *The Journal of Nutrition, 150*, 2588S—2592S.

Thesbjerg, M. N., Sundekilde, U. K., Poulsen, N. A., Larsen, L. B., & Nielsen, S. D. H. (2024). A novel proteomic approach for the identification and relative quantification of disulfide-bridges in the human milk proteome. *Journal of Proteomics, 301*(105), 194.

Tucci, M., Capparelli, R., Costa, A., & Rao, R. (1991). Molecular heterogeneity and genetics of *Vicia faba* seed storage proteins. *Theoretical and Applied Genetics, 81*, 50–58.

Vreeke, G. J., Meijers, M. G., Vincken, J. P., & Wierenga, P. A. (2023). Towards absolute quantification of protein genetic variants in *Pisum sativum* extracts. *Analytical Biochemistry, 665*(115), 048.

Wang, J., Suen, P. K., Xu, Z. F., & Jiang, L. (2009). A 64 kDa sucrose binding protein is membrane-associated and tonoplast-localized in developing mung bean seeds. *Journal of Experimental Botany, 60*(2), 629–639.

Warsame, A. O., Michael, N., O'Sullivan, D. M., & Tosi, P. (2020). Identification and quantification of major faba bean seed proteins. *Journal of Agricultural and Food Chemistry, 68*(32), 8535–8544.

Warsame, A. O., Michael, N., O'Sullivan, D. M., & Tosi, P. (2022). Seed development and protein accumulation patterns in faba bean (*Vicia faba*, L.). *Journal of Agricultural and Food Chemistry, 70*(30), 9295–9304.

Warsame, A. O., O'Sullivan, D. M., & Tosi, P. (2018). Seed storage proteins of faba bean (*Vicia faba* L): Current status and prospects for genetic improvement. *Journal of Agricultural and Food Chemistry, 66*(48), 12617–12626.

Wobus, U., Bäumlein, H., Bassüner, R., Heim, U., Jung, R., Müntz, K., & Weschke, W. (1986). Characteristics of two types of legumin genes in the field bean (*Vicia faba* L. var. minor) genome as revealed by cDNA analysis. *FEBS Letters, 201*(1), 74–80.

Wright, D. J., & Boulter, D. (1972). The characterisation of vicilin during seed development in *Vicia faba* (L.). *Planta, 105*, 60–65.