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Antioxidant peptides encrypted in flaxseed proteome: An *in silico* assessment

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ABSTRACT

Flaxseed proteins and antioxidant peptides (AP) encrypted in their sequences were analysed in silico with a range of bioinformatics tools to study their physicochemical properties, allergenicity, and toxicity. Nine proteases (digestive, plant and microbial sources) were assessed for their ability to release known APs from 23 mature flaxseed storage proteins using the BIOPEP database. The families of proteins identified were predominantly globulins, oleosins, and small amount of conlinin. Overall, 253 APs were identified from these proteins. More peptides were released by enzymatic hydrolysis from the globulins than those from oleosins and conlinin. Compared with other enzymes studied, the plant proteases (papain, ficin, and bromelain) were found to be superior to releasing APs from the flaxseed proteins. Analysis of toxicity by ToxinPred showed that none of the peptides released was toxic. Most of the APs showed structural features that are important for antioxidation, including relatively low molecular weight (dipeptides and tripeptides only); amphipathic properties (hydrophobicity range of – 0.5 to + 0.5); relatively low Boman index (≤2); broad range of pI (3.7–10.8), and an abundance of antioxidant amino acid residues (e.g. glutamic acid and histidine). This study demonstrate the suitability of flaxseed proteins as a source of APs.

1. Introduction

Oxidation of food components resulting in quality deterioration and generation of potentially toxic compounds is a challenge in the food and cosmeceutical industries [1,2]. Moreover, diseases caused by oxidative stress continue to be of worldwide concern as they have debilitating impacts on morbidity, mortality and overall quality of life of populations [3–5]. The aforementioned global challenges have sparked an interest in the development of various antioxidant compounds and ingredients for use in controlling oxidation both in food/cosmetic products and in human body systems. But the growing consumer preference for "natural" ingredients has meant that dietary intervention or use of food materials as a source of antioxidant peptides is preferred and often promoted over synthetic ingredients [3,6,7]. This is more so, considering that antioxidant peptides have the same mechanisms as other synthetic or natural antioxidants, acting as electron donors, radical trapping agents, pro-oxidant metal chelators, or activators of cellular redox enzymes [8].

Like other bioactive peptides, antioxidant peptides are inactive when present in their parent protein structure, and enzymatic hydrolysis (either *in vitro* or *in vivo*) is necessary to release them to exhibit their antioxidative activities [9]. The wide abundance of dietary proteins and proteolytic enzymes in nature means that protein-protease combinations that can generate peptides (with potential bioactivity) are almost limitless. This is promising, but the large dataset generated makes screening for peptides of desired activity a lengthy and laborious process.

The use of *in silico* or bioinformatics tools in the discovery of food-derived bioactive peptides overcomes this challenge by allowing researchers to select the most suitable protein and enzyme combinations that have the prospects of generating the desired bioactive peptides [10,11]. The outworking of these *in silico*

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tools is basically the same. Typically, the food protein sequences are selected from databases such as UniProtKB (https://www.uniprot.org/), Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep), or NCBI (https://www.ncbi.nlm.nih.gov/protein), followed by sequence alignment to compare and calculate commonality between the significant areas of protein sequences, to avoid repetition. The selected proteins are then subjected to *in silico* proteolysis, using tools such as ExPASy Peptide Cutter or BIOPEP "enzyme action", to generate peptides from conventional or novel proteolytic enzymes, or to find novel peptides from the protein sequences. These databases can also identify known bioactive peptides in the digested proteins, by matching the *in silico* dataset with published bioactive peptide sequences, and provide other useful parameters such as the ability of proteases to release certain peptides, or frequency of release of peptides with certain function. Ranking of these parameters helps identify the types of peptides, as well as the protein and protease sources that are most suitable for producing the bioactive peptides. The bioinformatics process therefore speeds up the discovery of bioactive peptides from food proteins.

In this study, flaxseed was analysed due to their relatively high protein contents (10%–37%) [12], nutritionally desirable amino acid profile [13], increasing reputation as a high-value 'superfood' [14], and the previously reported antioxidant capacity for its protein hydrolysates [15–17]. Moreover, a quantitative reference map of flaxseed proteins has been developed in previous studies [18–20], followed by a comprehensive proteomics dataset of flaxseed proteins, based on Phytozome database [21]. All these studies provide good proteomic information for further exploring the discovery of bioactive peptides from flaxseed proteins. The aim of this study was therefore to determine the physicochemical properties of storage proteins from mature flaxseeds, and comprehensively analyse the release and biochemical characteristics of antioxidant peptides from these proteins *in silico*.

2. Materials and methods

2.1. Source of flaxseed proteins

Barvkar et al. [21] identified about 1716 proteins in the proteome of flax (*Linum usitatissimum* L.). Only the storage proteins, which are more abundant in seeds and consumed as dietary proteins, found in matured seeds (48 days of anthesis, and ready for harvest) were selected for this study. Applying the exclusion criteria outlined in Fig. 1 resulted in the selection of only 23 proteins. The amino acid sequences of the selected proteins were then obtained from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html).

In silico proteolysis of mature flaxseed proteins with selected proteases

In silico proteolysis of the 23 mature flaxseed proteins was conducted with the 'enzyme(s) action' feature of BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) [22]. Analyses were done in April and May 2019. Out of 44 proteolytic enzymes available in BIOPEP, a total of nine (9) proteases representative of digestive ((trypsin (E.C. 3.4.21.4), pepsin (E.C. 3.4.23.1), pancreatic elastase II (E.C. 3.4.21.71)), plant ((papain (E.C. 3.4.22.2), stem bromelain (E.C. 3.4.22.32), ficin (E.C. 3.4.4.12)) and microbial proteases (thermolysin (E.C. 3.4.24.27), subtilisin (E.C. 3.4.21.62) proteinase P1 (lactocepin; E.C. 3.4.21.96)) were selected.

Prediction of antioxidant activity of peptides released after *in silico* enzymatic hydrolysis of flaxseed proteins

The release of antioxidant peptides from flaxseed proteins was predicted by opening the 'search for active fragments' tab of BIOPEP (after using the 'enzyme(s) action' application). The quantitative parameters of proteolysis were calculated, including the theoretical degree of hydrolysis (DH_t), the frequency of the release of fragments with a given activity by selected enzymes (A_E), and the relative frequency of the release of fragments with a given activity by selected enzymes (W). The formulae for calculating these parameters are given in Eq.s 1–3:

$$DH_t = \frac{d}{D} \times 100\% \quad (1)$$

where:

d – number of hydrolyzed peptide bonds

D - total number of peptide bonds in a protein chain

$$A_E = \frac{d}{N} \quad (2)$$

where:

d – number of fragments with specific bioactivity in a protein sequence that can be released by enzyme/s

N – number of amino acid residues of protein

$$W = \frac{A_E}{A} \quad (3)$$

where:

A_E – frequency of release of fragments with given activity by selected enzymes

A – frequency of occurrence of bioactive fragments in a protein sequence

Prediction of allergenicity of proteins, as well as solubility and potential toxicity of *in silico* derived antioxidant peptides

Potential allergencity of the flaxseed proteins was predicted by AllergenFP [23] (http://ddg-pharmfac.net/AllergenFP/). The solubility of peptides in water was estimated using Innovagen Peptide Solubility Calculator Proteomic Tool, available at https://pepcalc.com/peptide-solubility-calculator.php. ToxinPred [24] (https://webs.iiitd.edu.in/raghava/toxinpred/index.html) was used to predict the potential toxicity of the identified peptides.

2.2. Physicochemical properties of flaxseed proteins, and peptides released after in silico proteolysis

Several properties and indices of the 23 mature flaxseed proteins and *in silico*-derived antioxidant peptides were estimated using the application of 'Peptides' package [25] (version 2.4) in *R*, and/or ProtParam tool of ExPASy (https://web.expasy.org/protparam/). These properties included amino acid composition, amino acid length, molecular weight (MW), isoelectric point (pI), Boman index, net charge, hydrophobicity index (of peptides), and grand average of hydropathicity (GRAVY) values (of proteins).

3. Results and discussion

3.1. Classes of storage proteins found in flaxseeds

The 23 matured flaxseed proteins obtained from the work of Barvkar et al. [21] and used in this study corroborated what is expected in flaxseed, as reported in other studies [12,26,27]. The protein composition includes high-MW 11S globulin-type proteins (linin), a low-MW 2S albumin-type protein (conlinin), oil-binding proteins (oleosins), and glycoprotein precursors, which putatively are bound to the mucilage in flaxseed (see Table 1).

3.2. Physicochemical characteristics and indices of flaxseed storage proteins

Based on the primary sequence and MW, the matured storage proteins were divided into two groups: 7 low-MW proteins

Table 1
Physicochemical characteristics, indices and potential allergenicity of flaxseed storage proteins.

No.	Phytozome ID	Protein type	Protein description	GRAVY	MW (kDa)	pI	Boman index	Net charge	Allergenicity prediction	
									Potential allergenicity	Tanimoto similarity index with protein in database
1	Lus10031387	Oleosins	Oleosin low molecular weight isoform	0.25	15.40	10.1	0.64	4.27	Probable allergen	0.9 / NCBI # 198250343
2	Lus10017460		Oleosin low molecular weight isoform	0.03	16.31	10.6	0.99	6.52	Probable allergen	0.89 / NCBI # 5381321
3	Lus10039683		Oleosin low molecular weight isoform	0.24	15.98	9.7	0.50	3.27	Probable allergen	0.9 / NCBI # 198250343
4	Lus10027161		Oleosin low molecular weight isoform	0.22	16.18	9.8	0.57	3.45	Probable allergen	0.9 / NCBI # 198250343
5	Lus10014559		Oleosin high molecular weight isoform	0.09	18.71	9.9	0.64	5.39	Probable allergen	0.87 / NCBI # 10834827
6	Lus10028035		Oleosin 15.5 kDa	0.10	20.50	9.9	0.78	5.12	Probable non-allergen	0.86 / UniProtKB # P82251
7	Lus10003742		Oleosin 18.2 kDa	0.34	16.58	10.2	0.47	4.12	Probable allergen	0.87 / NCBI # 5381321
8	Lus10040396	Conlinin	Conlinin	−0.82	19.01	7.6	2.47	0.61	Probable allergen	0.81 / NCBI # 209165427
9	Lus10022929	Globulins	Legumin A	−0.60	52.97	7.6	2.37	0.68	Probable allergen	0.81 / UniProtKB # Q702P0
10	Lus10041224		7S globulin 2 precursor small subunit	0.08	48.39	8.1	0.98	4.35	Probable allergen	0.83 / NCBI # 3643813
11	Lus10024889		Legumin B precursor	−0.53	54.89	6.5	2.27	−4.59	Probable allergen	0.81 / NCBI # 62240392
12	Lus10003554		11S globulin seed storage protein	−0.54	55.20	5.6	2.02	−10.44	Probable allergen	0.82 / UniProtKB # Q9XHP0
13	Lus10011364		7S globulin	−0.21	50.92	5.8	1.58	−5.48	Probable non-allergen	0.84 / UniProtKB # Q96PE2
14	Lus10006420		7S globulin precursor	−0.24	51.29	5.7	1.64	−6.38	Probable allergen	0.86 / UniProtKB # Q9AVK8
15	Lus10010341		Glutelin type-A 3 precursor	−0.71	55.38	9.1	2.63	6.56	Probable allergen	0.84 / NCBI # 30313867
16	Lus10011817		11S globulin subunit beta precursor	−0.68	54.35	7.6	2.55	0.53	Probable allergen	0.84 / NCBI # 110349083
17	Lus10024891		Legumin B precursor	−0.52	54.39	6.6	2.29	−3.55	Probable allergen	0.84 / NCBI # 30313867
18	Lus10022927		Legumin B precursor	−0.52	54.47	6.4	2.28	−4.55	Probable allergen	0.83 / NCBI # 62240392
19	Lus10021179		Glutelin type-A 3 precursor	−0.77	55.84	7.6	2.68	0.69	Probable allergen	0.83 / NCBI # 307159114
20	Lus10033893		Legumin	−0.57	56.05	5.8	2.04	−9.38	Probable allergen	0.81 / UniProtKB # Q9XHP0
21	Lus10021180		11S globulin subunit beta precursor	−0.61	57.01	7.1	2.36	−1.44	Probable allergen	0.84 / NCBI # 156001070
22	Lus10042615		48-kDa glycoprotein precursor	−0.66	60.26	8.1	2.14	3.58	Probable allergen	0.84 / UniProtKB # Q8S4P9
23	Lus10022070		48-kDa glycoprotein precursor	−0.68	60.52	7.7	2.22	1.58	Probable allergen	0.85 / UniProtKB # Q8S4P9

Abbreviations: GRAVY, grand average of hydropathicity; MW, molecular weight; pI, isoelectric point.

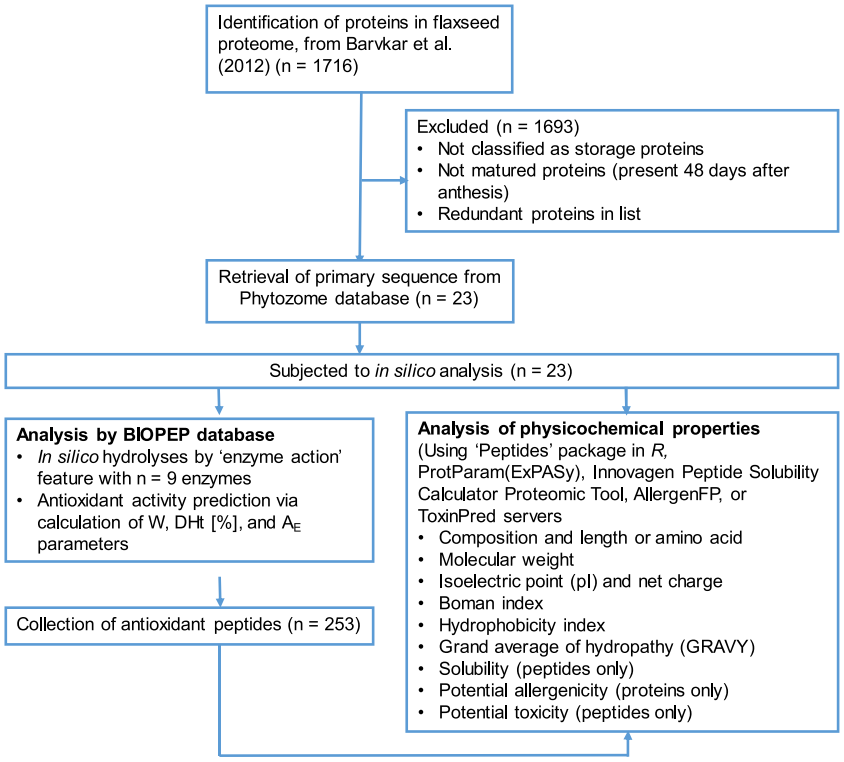

Fig. 1. Schematic of methods used in this study.

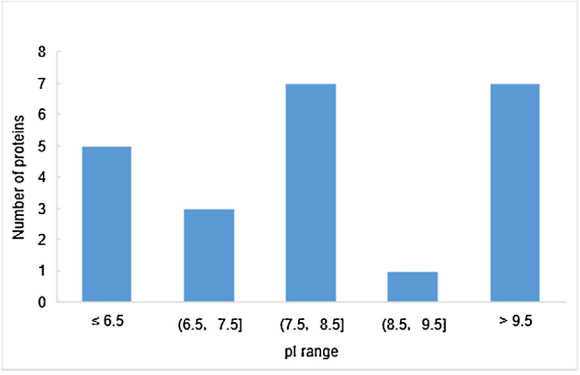

Fig. 2. Distribution of the isoelectric points (pI) of mature flaxseed storage proteins.

(15–20 kDa) and 16 high-MW proteins (48–60 kDa). Most of the low-MW proteins are oleosins, which account for about 2 – 8% of the total seed proteins in flaxseed and are responsible for stabilizing oil bodies in the seeds [28,29]. Conlinin (Lus10040396) was also identified as a low-MW protein (19 kDa, which is in the range of the 15–18 kDa value reported by Liu et al. [27]), and is the only 2S albumin identified in the matured flaxseed proteins used in this study. The high-MW proteins on the other hand are mainly storage proteins (legumin, glycoproteins, globulin, glutelin) and they make up more than 70%–85% of total flaxseed proteins [30].

The pI values of the mature flaxseed storage proteins are shown in Table 1 and summarized in Fig. 2. All the acid and neutral proteins (i.e., pI $\leq$ 6.5 and 6.5 < pI < 7.5, respectively) are predominantly globulins (legumins, 7S or 11S globulins) and albumins (conlinin). Other high-MW proteins, such as precursors of glutelin type A3 and 48 kDa glycoproteins, have pI greater than 8.0. On the other hand, the pI values of the oleosins are higher than 9.5 in each case (i.e., pI of 9.7–10.6), showing a marked predominance of positively charged (Arg, Lys, His) as opposed to negatively charged-amino acids (Asp, Glu). The calculated net charge of the proteins at pH 7 corroborated these results; in that, oleosins have net positive charge values (ranging from +3.27 to +6.52), and the net charge of other proteins ranged from -10.44 to +6.56. The high pI and positive net charge of oleosins is one of the structural features needed for the protein to interact electrostatically with negatively charged lipids, such as phospholipids, found in flaxseed oil bodies [28].

The proportion of non-polar and polar amino acids in the matured flaxseed proteins as well as the amino acid composition are summarized in Fig. 3A and B, respectively. Non-polar amino acids were more abundant in oleosins (~61%) than in the high-MW storage proteins (~47% and 50% in conlinin and globulins, respectively). The GRAVY values confirmed these results, with oleosins showing positive GRAVY of 0.03–0.34, and the high-MW storage proteins showing mostly negative (−0.24 to −0.77) GRAVY values (Table 1). The high hydrophobicity, or positive GRAVY values, observed for oleosins is expected since they are lipid-bound proteins and utilize their abundant non-polar amino acid residues (mostly Ala, Leu, Ile, and Val; see Fig. 3B) to interaction with and stabilize oil bodies in flaxseed [31]. Further, the near equal abundance of polar and non-polar amino acids in the globulins means that these proteins can have high amphipathic behaviour, which is desirable for surface-active functional properties such as foaming and emulsification. Nwachukwu and Aluko [32] recently demonstrated that globulins and albumins from flaxseed respectively exhibit foaming and emulsifying properties, due to high surface hydrophobicity (globulins) and high solubility (albumins).

The Boman index estimates the binding potential of a protein or peptide to a protein receptor. Thus, a peptide with high Boman index can exhibit multifunctional behaviour in being able to interact with a range of cytosolic proteins [33]. The Boman indices of oleosins range from 0.47 to 0.99, which demonstrate low potential for binding to other proteins. The Boman indices of the globulins ranged from 0.98 to 2.68, showing a high likelihood for these flaxseed proteins to bind to other proteins. This finding is expected

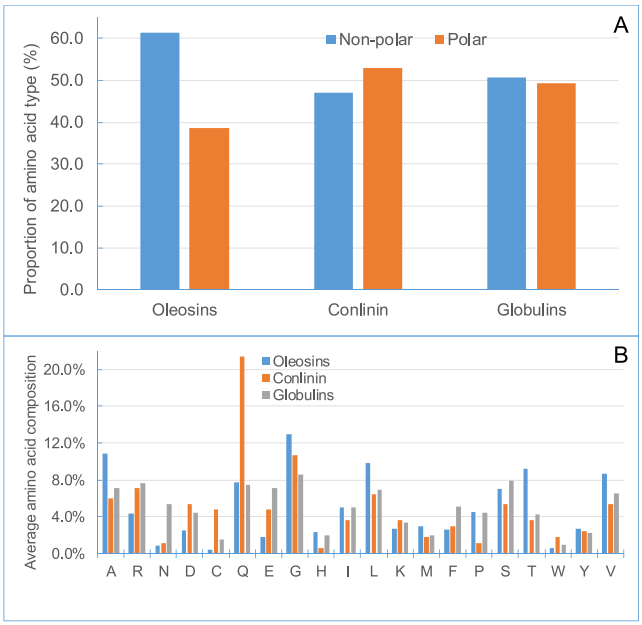

Fig. 3. (A) Proportion of non-polar and polar amino acids, and (B) average amino acid compositon in oleosins, conlinin and globulins. The proteins that make up each of the three classes (i.e., oleosins, conlinin and globulins) are presented in "Protein type" of Table 1. Amino acid codes: Alanine (A), Arginine (R), Asparagine (N), Aspartic acid (D), Cysteine (C), Glutamine (Q), Glutamic acid (E), Glycine (G), Histidine (H), Isoleucine (I), Leucine (L), Lysine (K), Methionine (M), Phenylalanine (F), Proline (P), Serine (S), Threonine (T), Tryptophan (W), Tyrosine (Y), and Valine (V).

as the oleosins, unlike the globulins, are naturally designed to bind to lipophilic oil bodies and are less capable of binding to other proteins in the seed. Thus, the Boman index of the flaxseed proteins is related to their hydrophobicity or GRAVY values.

The potential allergenicity of the flaxseed proteins, as estimated by AllergenFP, is shown in Table 1. AllergenFP is an alignment-free online platform for predicting allergenic proteins by transforming amino acid principal properties (e.g., hydrophobicity, size, abundance, hydrogen bonding behaviour (i.e., helix- and β-sheet-forming propensities)) into fingerprints that are used to predict allergenicity, via Tanimoto similarity searches with the allergenicity profile of known proteins [23]. The allergenicity prediction showed that all the proteins, except Lus10028035 (Oleosin 15.5 kDa) and Lus10011364 (7S globulin), are potentially allergenic, as they exhibited high Tanimoto indices (structural similarity) with some allergenic proteins reported in the literature (Table 1). Interestingly, although uncommon compared to the big 8 food allergens, a growing number of studies have identified flaxseed allergens. For example, Alpers and Sawyer-Morse [34] reported 5 allergens with MW of 38, 35, 30, 22, and 20 kDa; Lezaun et al. [35] identified a 56-kDa allergen, which would bind to IgE by forming disulphide bonds with intact polypeptides (150–175 kDa); Fremont et al. [36] identified an IgE-binding allergen (25–38 kDa), and estimated that there is flaxseed sensitization in 0.54%–1.08% of the French population. Alvarez-Perea et al. [37] reported a clinical case of flaxseed anaphylaxis in a 44-year-old man in Spain who presented dyspnoea and generalized urticaria after taking yogurt that contained flaxseed. The allergenic reaction was suspected to be caused by an 18-kDa IgE-binding allergen in the lipid soluble fraction of flaxseed, suggesting that the allergen could correspond to an oleosin. And recently, anaphylaxis reaction upon consumption of flaxseed, involving a 42-year-old female in Korea, has been reported, only 30 min after the subject consumed half teaspoon of flaxseed [38]. These evidences, together with the positive allergenicity predictions in our study, support the need to increase awareness of potential issues of allergenicity considering the rise in the consumption of flaxseed and its product.

3.3. Hydrolysis of the flaxseed proteins to generate peptides

3.3.1. Protease sources and effect on antioxidant descriptors

The average of frequency (A_E) and the number of antioxidant peptides released from mature flaxseeds proteins from each protease are shown in Table 2. The A_E values (0.0046–0.0052) and number of peptides released (48–59) by the plant proteases are significantly higher than those derived from the digestive enzymes (A_E of 0.0004–0.0015; number of peptides: 5–17). The relative frequency (W) of releasing antioxidant peptides by plant (0.0074–0.0332) and digestive (0.0929–0.1174) proteases were similar. This, combined with the fact that plant proteases released more peptides than the digestive proteases is an indication that the plant enzymes were more efficient at releasing antioxidant peptides, compared to the digestive enzyme. This also means that the production of antioxidant peptides from flaxseed proteins will potentially be more efficient during *in vitro* hydrolysis with plant proteases, rather than during physiological digestion by the action of digestive enzymes when flaxseed products are consumed. The differences in W for the various proteases can be explained by the difference in theoretical degree of hydrolysis (DHt), catalytic specificities, and number of recognition sites of each enzyme. The DHt when using the plant proteases (43.80% to 57.13%) are higher than those for digestive proteases (9.75% to 14.28%), and this is because the plant enzymes have a higher number of recognition sites (7 or 8) than the digestive enzymes (2 or 3)(see Table 2).

On the other hand, comparing the A_E, W and total number of peptides released shows that the efficiency of the microbial proteases is species dependent (Table 2). Subtilisin recorded the highest A_E (0.0061), W (0.1345) and number of peptides (58), whereas proteinase P1 gave the lowest A_E (0.0004), W (0.0126), and number of peptides (3). However, the theoretical degree of hydrolysis ([DHt]) of subtilisin (28.34%) is much lower than that of proteinase P1 (46.67%), suggesting that proteinase P1 released more peptide fragments from the proteins than subtilisin, but a low number of antioxidant fragments were generated. This result is due to the distinctive recognition sites of proteinase P1, which cuts both sides of leucine (L), proline (P), and glutamine (Q), potentially liberating single amino acid units. Leucine and proline are important amino acids in the list of antioxidant peptides identified in this study (see section 3.6). This means the potential antioxidant peptides hidden in mature flaxseeds protein sequences are more likely to be lost in the proteinase P1 digests, hence the release of only 3 antioxidant peptides by the protease.

Furthermore, as shown in Supplementary File 3, the number of antioxidant peptides released is also dependent on the protein type. The total number of antioxidant peptides released from the oleosins and conlinin is either one (1) or two (2), but the globulins yielded several peptides ranging from 8 to 28. By contrast, DHt of the oleosins and conlinin ranged from 30.30% to 35.74%, which are not substantially different from the DHt of the globulins (29.45%–33.99%). This finding suggests that the globulins of flaxseed are better precursors of antioxidant peptides than the oleosins and conlinins. This observation is attributable to the differences in primary sequence of the protein classes. The globular proteins have higher numbers of amino acid residues (473–535), and so have a greater chance of generating more bioactive peptides. The oleosins and conlinin on the other hand are relatively smaller proteins with 146–198 residues.

Correlation of protein properties with W, relative frequency of the release of antioxidant peptide by selected enzymes

In an attempt to relate the properties of the proteins with enzyme specificity and likelihood of release of antioxidant pep-

Table 2
Enzyme types and their *in silico* hydrolytic performance.

Enzyme Source	Enzyme Type	Recognition site in protein sequence (from BIOPEP database)ss	Total number of peptides	Average [DHt] (%)	Average A_E	Average W
Digestive	Trypsin	K*, R*	17	9.75	0.0015	0.0332
	Pepsin	F*, L*	5	12.02	0.0004	0.0074
	Pancreatic elastase II	L*, M*, F*	5	14.28	0.0004	0.0074
Plant	Papain	R*, F*, *Q, L*, G*, T*, *A	48	45.50	0.0046	0.0929
	Ficin	F*, Y*, G*, S*, L*, R*, H*	51	43.80	0.0048	0.1174
	Stem bromelain	V*, A*, T*, L*, R*, G*, S*, F*	59	57.13	0.0052	0.1022
Microbial	Thermolysin	*A, *L, *I, *V, *Y, *F	37	34.66	0.0034	0.0703
	Proteinase P1 (Lactocepin)	*Q*, V*, *P*, *S, *L*	3	46.67	0.0004	0.0126
	Subtilisin	Y*, F*, L*, *V, W*, S*	58	28.34	0.0061	0.1345

*indicates cutting position of enzyme; e.g. *X and X* means the enzyme cuts at N- and C-terminal, respectively, and *X* means the enzyme cuts at both sides of the amino acid.

Table 3
Correlation of protein properties with W.

Enzyme Source	Enzyme Type	GRAVY	MW	pI	Boman index	Net charge
Digestive	Trypsin	−59%	61%	−44%	69%	−30%
	Pepsin	−19%	40%	−47%	14%	−41%
	Pancreatic elastase II	−19%	40%	−47%	14%	−41%
Plant	Papain	−26%	44%	−58%	24%	−70%
	Ficin	17%	9%	−8%	−15%	−14%
	Stem bromelain	−60%	77%	−76%	66%	−68%
	Thermolysin	−83%	74%	−53%	85%	−22%
Microbial	Proteinase P1 (Lactocepin)	14%	−20%	28%	−18%	29%
	Subtilisin	0%	12%	−28%	5%	−29%

tides, the Pearson correlation coefficients was computed between protein properties and W for each enzyme. As shown in Table 3, GRAVY and Boman index of proteins each had significant linear relationships with W for thermolysin (i.e., $r = -83\%$ and 85%, respectively). The MW of proteins were also significantly correlated to the W of stem bromelain ($r = 77\%$) and thermolysin ($r = 74\%$). The pI and net charge of proteins were also significantly correlated to the W for stem bromelain ($r = -76\%$) and papain ($r = -68\%$), respectively.

3.4. *Physicochemical characteristics of the* in silico *antioxidant flaxseed peptides*

The physicochemical characteristics of the *in silico*-derived antioxidant peptides were calculated and presented in Fig. 4A. The MW of the antioxidant peptides ranged from 226 to 455 Da. The dipeptides had MW ranging from 226 to 317 Da, with an average of 272 Da; and tripeptides had MW range of 346 to 455 Da, with an average of 390 Da. As shown in Fig. 4, dipeptides with MW in 250–275 Da and 275–300 Da range were more abundant, whereas tripeptides with MW in the range 350–375 Da were the most abundant.

Out of the 253 antioxidant peptides obtained from the flaxseed proteins, 63 had pI $\leq$ 4.5, with net charge of -1.00. These peptides usually contained Glu (E) in their primary sequence. About 63 peptides had 5.5 < pI $\leq$ 6.5, and 48 had 7.5 < pI $\leq$ 8.5, with net charges ranging from -0.06 to 0.18. Moreover, 79 peptides had pI > 9.5 and, out of these peptides, 77 had net charges that ranged from 1.00 to 1.06; the latter all had Lys (K) or Arg (R) in their primary structure. The remaining two (2) peptides had the net charges of 2.00, having two Lys (K) groups each (Fig. 4 B, D). It can be concluded that about 50% of the antioxidant flaxseed peptides were acid and negatively charged, and another 50% were basic and positively charged.

The Boman indices of the antioxidant flaxseed peptides ranged from −3.625 to 7.38 (Fig. 4 C). The Boman index of most of the peptides (about 189 peptides) were less than 2. However, 64 peptides showed Boman indices > 2, and these peptides have a greater likelihood to bind to several proteins within a cell or may exhibit multifunctional behaviour.

The hydrophobicity of the peptides ranged from -3.70 to 2.65 (Fig. 4E). There were hydrophilic peptides (about 36, with hydrophobicity of between –3.70 and –0.50) as well as hydrophobic peptides (about 43, with hydrophobicity between 0.50 and 2.65). Amphipathic peptides with hydrophobicity values between −0.50 and 0.50 were predominant (about 174). The water solubility potential of the peptides, as predicted by Innovagen's Peptide solubility calculator are shown in Table 4. In line with the wide hydrophobicity range of the peptides, there was a balanced distribution of both water-soluble and water-insoluble peptides. The varying hydrophobicity and solubility profiles of the flaxseed peptides show that they can function in a range of systems, whether water-soluble, lipid-soluble, or emulsion-based.

3.5. *Potential toxicity of released flaxseed peptides*

This study was focused on the discovery of peptides with potential antioxidant properties. As an *in silico* study, there is the likelihood of identifying novel peptides. Therefore, it is necessary that all the peptides released from the flaxseed proteins be subjected to potential toxicity evaluation. Toxicity prediction was undertaken by ToxinPred [39], which uses a hybrid model based on dipeptide composition and motif scanning to predict the toxicity of peptides or identify toxic regions in a protein. In this instance, none of the antioxidant peptides released from flaxseed proteins *in silico* (see Table 4) had potential toxicity, suggesting that they are suit-

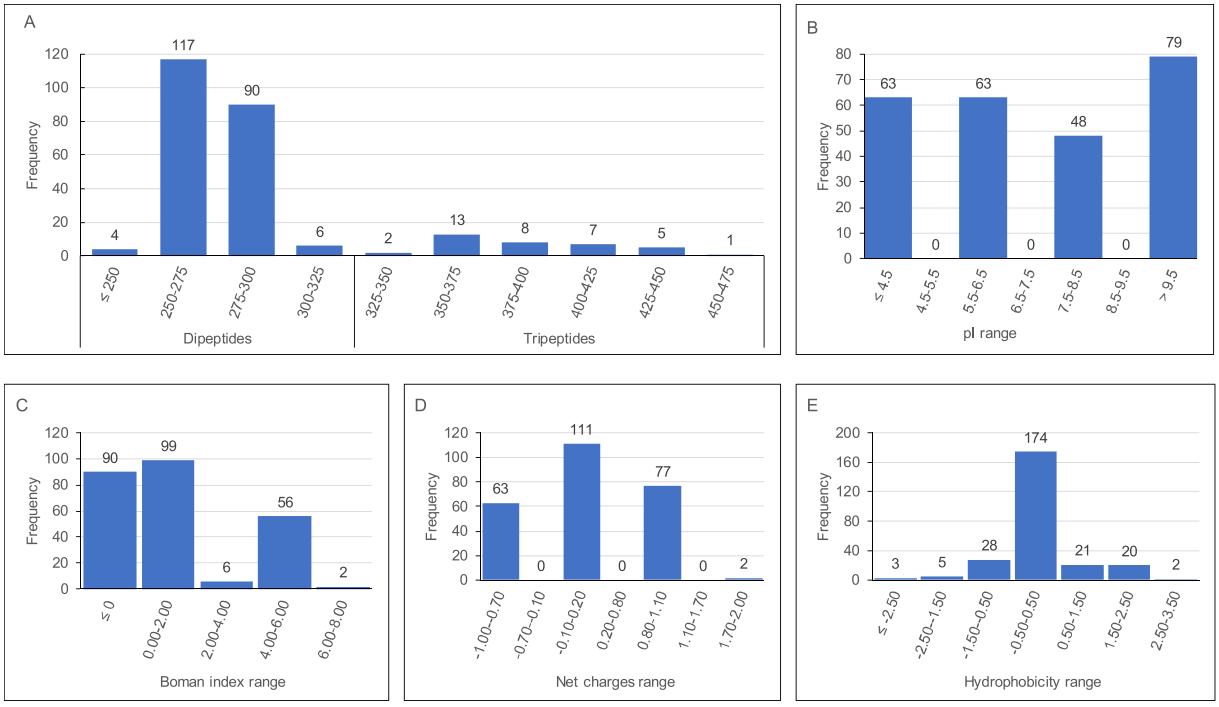

Fig. 4. Distribution of physicochemical properties (A. molecular weight; B. pI; C. Boman index; D. net charge; and E. hydrophobicity index) of the antioxidant peptides released from flaxseed proteins *in silico*.

Table 4
Sequences, predominance and toxicity prediction of antioxidant peptides identified in matured flaxseed proteins.

		ToxinPred output		
Peptide	Number of occurrences	Support Vector Machine score	Toxicity prediction	Predicted solubility in water
ADF	7	−0.84	Non-Toxin	Good
AH	4	−0.8	Non-Toxin	Poor
AW	1	−0.8	Non-Toxin	Poor
AY	2	−0.8	Non-Toxin	Poor
EAK	2	−0.75	Non-Toxin	Good
EL	51	−0.8	Non-Toxin	Good
FC	2	−0.8	Non-Toxin	Poor
HHY	1	−0.82	Non-Toxin	Poor
HL	30	−0.8	Non-Toxin	Poor
IKK	2	−0.83	Non-Toxin	Good
IR	51	−0.8	Non-Toxin	Good
IY	10	−0.8	Non-Toxin	Poor
KD	1	−0.8	Non-Toxin	Good
LH	3	−0.8	Non-Toxin	Poor
LHQ	1	−0.81	Non-Toxin	Poor
LHS	1	−0.82	Non-Toxin	Poor
LK	20	−0.8	Non-Toxin	Good
LW	1	−0.79	Non-Toxin	Poor
LWE	2	−0.76	Non-Toxin	Good
MHH	1	−0.82	Non-Toxin	Poor
MHI	1	−0.81	Non-Toxin	Poor
MM	4	−0.8	Non-Toxin	Poor
MY	1	−0.8	Non-Toxin	Poor
PEL	3	−0.84	Non-Toxin	Good
PHF	2	−0.82	Non-Toxin	Poor
PHV	2	−0.83	Non-Toxin	Poor
PHW	2	−0.8	Non-Toxin	Poor
RHL	5	−0.78	Non-Toxin	Good
RHT	1	−0.79	Non-Toxin	Good
TY	5	−0.8	Non-Toxin	Poor
VW	4	−0.8	Non-Toxin	Poor
VY	19	−0.8	Non-Toxin	Poor
VYV	2	−0.84	Non-Toxin	Poor
WG	8	−0.8	Non-Toxin	Poor
YYS	1	−0.8	Non-Toxin	Poor

Table 5
Amino acid composition of all 253 antioxidant peptides released from flaxseed proteins *in silico*.

Amino acid	Amino acid content (%)	Number of peptides containing given amino acid
Ala (A)	3.0	16
Arg (R)	10.5	57
Asn (N)	0.0	0
Asp (D)	1.5	8
Cys (C)	0.4	2
Gln (Q)	0.2	1
Glu (E)	10.7	58
Gly (G)	1.5	8
His (H)	10.3	56
Ile (I)	11.8	64
Leu (L)	21.6	117
Lys (K)	5.0	25
Met (M)	2.0	11
Phe (F)	2.0	11
Pro (P)	1.7	9
Ser (S)	0.4	2
Thr (T)	1.1	6
Trp (W)	3.3	18
Tyr (Y)	7.7	42
Val (V)	5.4	29

able for further development and use in food and pharmaceutical products.

Structure-activity relationship between key amino acids of the *in silico* peptides and antioxidant property

Table 5 shows the sequences and number of *in silico* antioxidant peptides released from flaxseed proteins, totalling 253 antioxidant peptides (217 dipeptides and 36 tripeptides). The antioxidant peptides with short size (<3 kDa) are usually believed to have higher lipid peroxidation inhibition and scavenging activities [40]. However, the antioxidant activities (radical scavenging, reducing power, or metal chelation) are not only dependent on peptide size, but also on amino acid composition [41]. Table 5 shows the amino acid composition of the peptides. Antioxidant flaxseed peptides contain an abundance of hydrophobic amino acids such as Leu (L), Ile (I), and Val (V) (21.6%, 11.8% and 5.4%, respectively). These hydrophobic amino acids in antioxidant peptides are thought to promote antioxidant properties by increasing the solubility of peptides in the lipid phase of oxidation systems. When they occur *in situ*, in biological or food systems, they provide protection against lipid peroxidation and cellular damage through interacting with cell membranes or inhibiting lipid peroxidation at the water-lipid interface [42,43]. On the other hand, the hydrophobic amino acid at the N- terminal could facilitate the interaction between fatty acids and peptides, thereby increasing the proximity of peptides to lipophilic intermediate radicals and, hence, enhancing free radical quenching [44].

Furthermore, there is an abundance of the negatively charged amino acid, Glu (E) (10.7%), in the identified antioxidant peptides. This amino acid has the ability to scavenge free radicals and enable reduction of ferric ions by donating electron, and may also bind to transitional metal ions and change redox cycling capacity, thereby inhibiting the propagation of lipid peroxidation [42].

Another important group of amino acids present in the flaxseed antioxidant peptides is the aromatic amino acids, Tyr (Y), Trp (W) and Phe (F) (7.7%, 3.3%, 2.0%, respectively), as well as His (H) (10.3%). Most of the Tyr residues are located at the C-terminal of the antioxidant flaxseed peptides. The aromatic rings in Tyr has pro-oxidant metal ion chelation and free radical (e.g. ROS) scavenging properties [41,42,45]. Histidine (H), mainly found at the N-terminal or middle of the antioxidant peptides, also have both hydroxyl radical scavenging and active oxygen radical quenching activities, together with an ability to use its imidazole ring to chelate metal ions [46]. A previous study showed that a large amount of His and hydrophobic amino acids in dairy whey peptides contribute to lipid oxidation inhibition [47]. Trp (W) and Phe (F) also have the abilities to scavenge radicals and chelate metal ions [42,43], but their relative abundance is low in the antioxidant flax peptides identified in this study. It is noted that the Phe would have negative effect on inhibition of hydroperoxide formation compared to Trp and Tyr, due to lack of indole and hydroxyl group [48]. There was also a good representation of Arg (R) and Lys (K) in the antioxidant peptides, but these cationic amino acids are not antioxidative and their role in the antioxidant peptides is not clear.

4. Conclusion

In this study, we presented the physicochemical properties of 23 mature flaxseed storage proteins, as well as their potential to serve as precursors of antioxidant peptides, upon enzymatic digestion. According to MW, the proteins are separated into two groups, oleosins (15.40–20.50 kDa) and high-MW proteins consisting of conlinin and globulins (48.39–60.52 kDa). All the oleosins had similar chemical properties such as higher pI, positive net charge, lower Boman indeces, and positive GRAVY, suggesting higher hydrophobicity. The high-MW proteins had lower pI values, wider range of net charge, higher Boman indices, and negative GRAVY. Based on the number of peptides released, A_E and W, plant enzymes (ficin, bromelain and papain) have higher potential than digestive enzymes (trypsin, pepsin and pancreatic elastase II) in releasing antioxidant peptides from the flaxseed storage proteins. The potential for peptide release from the microbial enzymes is species dependent, and proteases such as subtilisin released the highest number of peptides (58 peptides) and proteinase P1 releasing the least (3 peptides). Correlation of protein properties with the relative frequency of release of antioxidant peptides showed several significant linear relationships between GRAVY, protein Boman index and W for thermolysin, between protein MW and W for bromelain and thermolysin, and between pI values, protein net charge and W for stem bromelain and papain.

Physicochemical characteristics of the antioxidant peptides from flaxseed storage proteins showed that the identified peptides are dipeptides and tripeptides with a balanced distribution of acidic and basic peptides (based on net charge), as well as a wide range of hydrophobicity, Boman indices and varying profile of potential solubility in water. These findings, together with the prediction that the peptides are potentially non-toxic, suggest that the identified peptides have strong prospects for use in hydrophilic and hydrophobic food systems. Finally, hydrophobic (L, I), negatively charged (E), aromatic (Y) amino acids, and His (H) more frequently occurred in the peptides, and therefore must be critical for the antioxidant properties. Flaxseed storage proteins are therefore competitive feedstock for the production of antioxidant peptides. Follow up studies involving wet synthesis and evaluation of antioxidant capacity are needed to confirm the *in silico* findings.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fshw.2019.08.002.

Declaration of Competing Interest

All authors declare that they have no competing interests.

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