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FUNCTIONAL PROPERTIES OF FABA BEAN PROTEIN AND EFFECT OF ENZYMATIC HYDROLYSIS ON ITS ANTIOXIDANT ACTIVITY

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ABSTRACT: Protein play an important role in determining structure and texture of various food products. Therefore, the goals of this research were to study the functional properties of faba bean protein cultivar, Giza3, compared to ß-Lactoglobulin (ß-Lg) and to hydrolyze this protein using pepsin at different pH values (1.5 and 3) and different incubation periods (0, 5, 10, 60, and 180 min) to characterize the resultant hydrolysates and evaluate their antioxidant activities. The solubility at different pH, emulsifying properties, stability against creaming and oil droplets size, of faba bean protein were tested compared to ß-Lg protein. The findings cleared that the solubility and emulsifying properties of faba protein were very low compared to ß-Lg. Concerning to hydrolysis process, the degree of hydrolysis at pH 1.5 was higher than that at pH 3. The molecular weight distribution bands of faba protein hydrolysates were in the range of 9–98 kDa using SDS-PAGE method while, peptides were in the range of 500 – 4000 Da using MALDI-TOF MS method. The results of both methods confirmed that the hydrolysis at pH 1.5 was higher than pH 3. Moreover, the enzymatic hydrolysis significantly improved the antioxidant activity of faba bean protein. Hydrolysates produced at pH 3 had a slight high antioxidant activity than at pH 1.5 at all incubation periods. Finally, these results suggest that faba bean hydrolysates could be used in preparing functional foods and as natural antioxidants to prevent oxidation process in food products.

Key words: Faba bean protein, functional properties, enzymatic hydrolysis, SDS-PAGE, MALDI-TOF MS, antioxidant activity.

INTRODUCTION

Faba bean (FB), also known as *Vicia faba*, fava bean or broad bean, is a flowering plant in the family of Fabaceae and genus of *Vicia*. In Egypt, the cultivated area and amount of production of faba beans decreased from 221000 faddan and 413 tons in year 2005 to 88000 faddan and 142 tons in 2016 (**Egyptian Ministry of Agriculture and Land Reclamation, the Economic affairs Sector)**. It is one of the main sources of inexpensive protein in Africa, Latin America and parts of Asia **(Haciseferogullari *et al.,* 2003).** 7S and 11S globulins, albumins and glutelins are the main fractions of FB proteins **(Hossain and Mortuza, 2006; Kimura *et al.,* 2008)**. When the anti-nutritional factors content is low in the FB meal, it could be used well as a good source for amino acids, especially lysine, for supplementing the cereals and for feeding the laying hens **(El-Fiel *et al.,* 2002; Magoda and Gous, 2011)**. The FB protein isolate showed the lowest solubility at a range of pH from 4 to 6, while it showed the highest solubility at pH 8 and 9 **(Fernández-Quintela *et al.,* 1997)**.

Although several animal proteins showed excellent organoleptic and functional properties, unluckily, they require a high production cost compared to plant proteins. The problem is that the functionality of plant protein is often unsatisfactory due to the low solubility in aqueous media. Several studies evaluated the functionality of plant proteins (chickpeas, lupins, lentils, … *etc*.) **(Bamdad *et al.,* 2006;**

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Morales-De Leon *et al.*, 2007). Enzymatic hydrolysis intensively improves the solubility of proteins and modifies their functional properties, depending on three main structural changes: a reduction of mole mass, a greater availability of hydrophobic regions and the release of ionizable groups. The enzymatic hydrolysis was done on the protein of Red Tilapia fish using thermolysin and alcalase enzymes under optimum conditions. The results showed that both enzymatic hydrolysates yielded an increase in peptide content and antioxidant activity **(Daud *et al.*, 2013)**.

Alkali or acid protein hydrolysis compared to enzymatic hydrolysis offers more moderate conditions of the process and few undesirable products. In the enzymatic hydrolysis, the functionality of hydrolysate can be controlled by selection of specific enzymes, which used to break specific peptide bonds, and reaction factors. The resulted peptides have a small molecular size compared to normal proteins. Therefore, their functional properties are changed: improved solubility at different pH values, reduced viscosity, and noteworthy changes in emulsifying, gelling, and foaming properties **(Hrckova *et al.*, 2002)**.

The functional properties, such as antioxidant activity, of different proteins including soybean and milk have also modified using enzymatic hydrolysis **(Le Tien *et al.*, 2001; Hu *et al.*, 2003; Penas *et al.*, 2004; Chove *et al.*, 2007)**. Therefore, this research was undertaken for four objectives: (i) study the functional properties of faba bean protein compared to ß-Lactoglobulin (ß-Lg) (ii) hydrolysis of faba bean protein using pepsin enzyme and evaluate whether the resultant protein was affected by time and pH conditions (iii) characterization of protein hydrolysates using RP-HPLC and SDS-PAGE methods in addition to MALDI-TOF MS, and finally (iiii) to determine the antioxidant activity of resulted hydrolysates.

MATERIALS AND METHODS

Materials

Faba bean Giza 3 cultivar was obtained from Agriculture Research Center, Institute of Field Crops, Giza, Egypt. ß-Lg powder was bought from Davisco Foods International, Inc. USA. Pepsin (EC 3.4.21.1) was obtained from Sigma-Aldrich Co. Ltd. Coomassie Brilliant Blue R-250 and bovine serum albumin (BSA) were bought from Serva (Heidelberg, Germany), and 2,5-Dihydroxy actetophenone (DHAP) was obtained from Bruker Daltonik GmbH, Germany. The other reagents and chemicals were in an analytical grade.

Methods

Sample Preparation

Faba beans were milled by an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Germany), with a 0.4 mm mesh ring sieve. The milled samples were kept at -20˚C in polyethylene bags till analysis.

Determination of Gross Chemical Composition of Faba Bean

The chemical composition of FB flour (moisture, protein, fat, fiber, ash, and available carbohydrates calculated by difference) was determined using Near Infrared spectrometry (NIR) (Unity Scientific Co, USA) included InfoStar Software, version 3.11.1 **(AOAC, 2011; Wimonsiri *et al.*, 2017)**.

Extraction of Faba bean Protein

Faba bean protein was extracted using 0.04% ascorbic acid solvent as described by **Ali *et al.* (2012)**. Two grams of frozen FB flour were placed in 50 ml centrifuge tube. Then 20 ml ascorbic acid (0.04%) was added. After that, the mixture was stirred at room temperature for 4 hr., and centrifuged at 4100 × g for twenty minutes, then the clear supernatant was carefully collected, freeze dried. Protein was kept at –20°C in plastic bottles until analysis.

Determination of Protein Content of Extracts

Protein content was determined according to the method of **Bradford (1976)** using BSA as a standard.

Functional Properties of Extracted Protein

Solubility of faba bean protein compared to ß-Lg protein

One milligram FB and ß-Lg proteins were dissolved in one milligram of sodium phosphate

buffer (0.05 M) at different pH values (2 - 9). Protein solutions were centrifuged at 9250 × g and 4°C for 10 min and the protein content of supernatants was measured according to the method of **Bradford (1976)**.

Emulsifying Properties of Faba Bean Protein Compared to ß-Lg Protein

Preparation of emulsions

FB and ß-Lg protein emulsions (10% oil in distilled water) were prepared as mentioned by **Khalil *et al.* (2012)** with some modifications. 0.2% protein solution was stirred with the oil at 500 rpm for ten minutes, then the emulsifying process was completed by an ultrasonic homogenizer Sonicator (Bandelin GmbH. Germany) for five minutes.

Oil droplets size of emulsion

Oil droplets size of emulsions and their distribution were estimated by Malvern Mastersizer S, Germany. The volume weighted mean of oil droplet size D (4,3), the surface weighted mean diameter of oil droplet D (3,2), and average size of oil droplets (10^{th}, 50^{th} and 90^{th} percentile of the diameter) were calculated by the software of equipment depending on Mie's theory **(Khalil *et al.*, 2012)**.

Stability of emulsion against creaming

After homogenization, the stability of emulsions against creaming was measured under centrifugal force as mentioned by **Ali *et al.* (2013)** using a spectrophotometer (SPEKOL, Carl Zeiss, Germany).

Enzymatic hydrolysis of faba bean protein

Enzymatic hydrolysis process was done according to **Yang *et al.* (2011)**, with slight modifications. Forty grams of FB flour were mixed with 400 ml ascorbic acid (0.04%). Next, the mixture was stirred at room temperature for 4 hr., and centrifuged at 4100 × g for 20 min, after that the supernatants were carefully collected and the pH was adjusted with 1N HCl to 1.5 and 3.0 and temperature at 34°C. Then the pepsin was added, ratio of enzyme to protein was 1:100 (*W/W*). Samples were removed after 0, 5, 10, 60 and 180 min and adjusted to pH 7.5-8 to inactivate the enzyme. The hydrolysates were then freeze-dried and ground for further analysis.

Degree of hydrolysis

The degree of hydrolysis (DH) was calculated as the ratio between the content of protein after hydrolysis and the total protein content before hydrolysis **(Bradford, 1976)**.

Determination of free amino groups content of hydrolysates by TNBS method

Trinitrobenzenesulfonic acid (TNBS) method was used to analyze the content of free amino groups in faba bean hydrolysates according to **Adler-Nissen (1979)**. Protein solution (mg/in ml 1% sodium dodecyl sulfate, SDS) was kept with TNBS substance at pH 9 and 40°C for 60 min then, the absorbance of resulted colour was recorded at 340 nm by Pharmacia Biotech spectrophotometer, England. Isoleucine in range from 20 to100 nM was used to prepare the calibration curve.

Determination of protein content of hydrolysates using RP-HPLC

Reversed phase–high performance liquid chromatography method was used to determine the protein content. Four milligrams of faba protein and hydrolysates were dissolved in one ml of potassium phosphate (PBS) buffer at pH 7.2, then analyzed by Shimadzu HPLC system (Kyoto, Japan) using a Perfectsil 300 – C 8 column (300 x 4,6 mm; 5 µm) at 50°C. HPLC grade water containing 0.1% (*V/V*) triflouroacetic acid (TFA) was the eluent A and acetonitrile was eluent B. The injection volume was 100 µl and the flow rate was 1 ml/min. The gradient was applied as mentioned by **Ali *et al.* (2018)** and the detection wavelength was 280 nm.

Determination of hydrolysates molecular weight using SDS-PAGE technique

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to **Laemmli (1970)** was used to determine the molecular weight of hydrolysates. FB protein and hydrolysates were denaturized by heating at 95°C for five minutes in SDS buffer (0.0625 mol/l Tris-HCl buffer (pH 6.8) with 5% 2-mercaptoethanol, 0.002% bromophenol blue, 10% glycerol and 2% SDS). The gel was immersed in a Coomassie blue solution for two hours followed by a discoloration solution

(water/methanol/acetic acid, 5.2/4/0.8, *V/V/V*) for 12 hr., Sigma Marker ™ Low Range of 14–96 KDa molecular range was utilized as a standard. Quantity One Software (Bio-Rad Laboratories, Italy) was used to estimate the molecular weight and intensity of protein band.

Determination of hydrolysates molecular weight using MALDI-TOF MS technique

Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI- TOF MS) technique based on **(Perkins *et al.,* 1999; Ali *et al.,* 2013)** was used to determine the molecular weight of hydrolysates. One milligram of native FB protein and hydrolysates were dissolved in one ml of 0.1% trifluoroacetic acid/ acetonitrile (50%, *V/V*). Briefly, 0.5 µl of each solution was transported on the MALDI plate and covered with 0.5 µl DHAP matrix, and left to dry at room temperature. After crystallization of the sample, measurements were achieved on AUTOFLEX-III LRF200-CID, equipped with Smartbeam-Laser 200 (Bruker Daltonik GmbH, Germany) and the molecular weight of hydrolysates was estimated using the Bruker Daltonics Flex Analysis software**.**

Determination of antioxidant activity of hydrolysates by TEAC assay

Antioxidant activities of native FB protein and hydrolysates were tested by trolox equivalent antioxidant capacity assay (TEAC) as described by **Ali and Elsharkawy (2018)**, using a Pharmacia Biotech spectrophotometer, England. The results are calculated as µM TE/mg protein.

Statistical Analysis

The obtained data were statistically analyzed using SPSS software version 18. Values of $P \leq 0.05$ was considered statistically significant **(Kumar *et al.,* 2016; Ali and Elsebaie, 2018)**.

RESULTS AND DISCUSSION

Chemical Characteristics of Faba Beans

The chemical composition of FB flour was determined and the obtained results are presented in Table 1. Results indicated that the flour contained 13.32, 37.33, 3.00, 7.81, 2.97 and 48.89%, for moisture, protein, crude fat, crude fiber, ash and available carbohydrates, respectively. Concerning the crude protein content, results disclose that the FB flour contained high amount of crude protein. The value of protein is lower than the findings values obtained previous **(Haciseferogullari *et al.,* 2003; Alghamdi, 2009; Hendawey and Younes, 2013)**. They reported that, the content of protein greatly varied according to faba bean cultivars. Moreover, these variations may be also related to the genetic, fertilization, and environmental factors. On the other hand, it is in the line with the results of **Musallam *et al.* (2004), Hossain and Mortuza (2006) and Kumar *et al.* (2015).** Therefore, FB protein can be used as protein supplement to cereal based foods or can provide a good protein source to produce weaning foods of high nutritive value. Moreover, it could contribute effectively to alleviate the protein malnutrition problem in the developing countries. The percentage of moisture, fat, fiber, and carbohydrates of different cultivars of FB was in a range (7.05 to 8.18), (1.5 to 2.20), (4.61 to 6.91), and (42.2 to 47.3), respectively **(Alghamdi, 2009; Hendawey and Younes, 2013)**.

Solubility of Faba bean Protein Compared to ß-Lg

The solubility is consider one of the main functional properties of protein and is very important for food products. The solubility of FB protein, at different pH values, compared to ß-Lg was measured and the results are shown in Fig. 1. The solubility of FB protein at pH 2 to 9 was significantly lower than ß-Lg especially at pH between 3 and 6, where it ranged from 53.5 to 58.8% for FB protein while, it ranged from 83.2 to 94.1% for ß-Lg. Results also showed that the solubility was higher at pH (7 to 9) where, the values were ranged from 81.3 to 88.1% but still lower than ß-Lg protein.

Emulsifying Properties of Faba Bean Protein Compared to ß-Lg Protein

Oil droplets size

Faba bean protein was used as emulsifier and the emulsifying properties measured directly after the emulsification and compared with ß-Lg protein (Fig. 2). The results of 10^{th} percentile of

Table 1. Chemical composition (% on dry weight basis) of faba bean flour

Constituents	Moisture	Protein	Crude fat	Crude fiber	Ash	Available carbohydrates
(%)	13.32±0.3	37.33±8.67	3.00±0.03	7.81±0.13	2.97±0.65	48.89±8.25

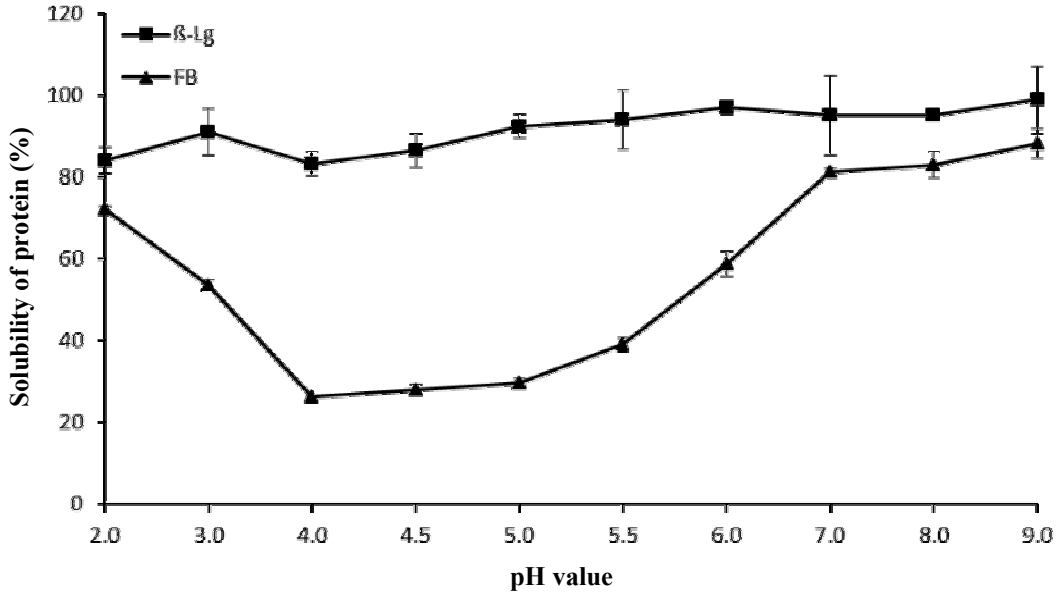

Fig. 1. Solubility of faba protein at different pH values compared to ß-Lg

Where: ß-Lg, Beta-Lactoglobulin and FB, Faba bean protein

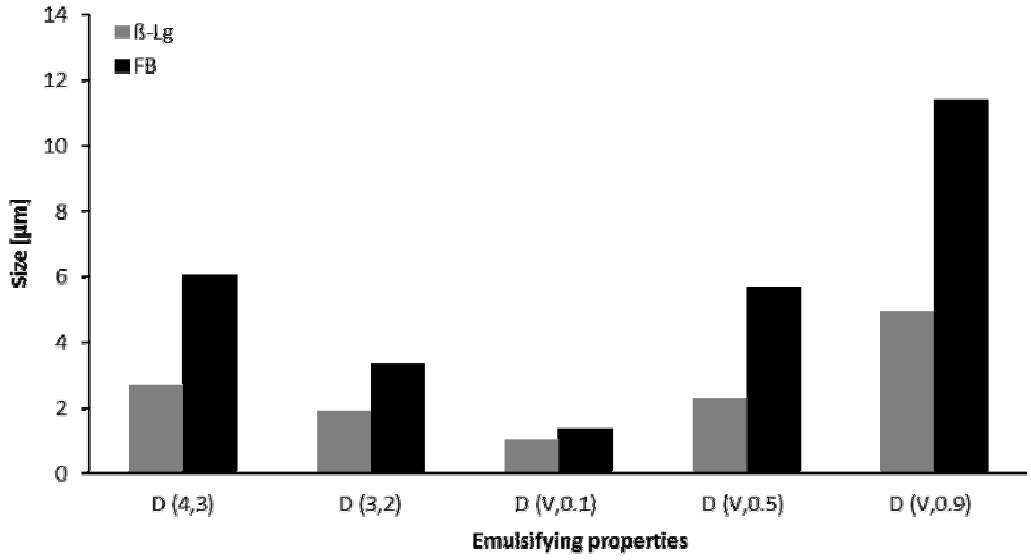

Fig. 2. Oil droplets size of emulsions prepared with faba bean protein compared to ß-Lg

Where: ß-Lg, Beta-Lactoglobulin and FB, Faba bean proteins.

particles size D (v, 0.1) showed slight differences between emulsions of FB and ß-Lg proteins. In contrast, significant differences were reported between them in the 50th percentile of particles size D (V, 0.5) and 90th percentile of the particle size (V, 0.9). Moreover, it was observed from Fig. 2 that, significant differences ($p \leq 0.05$) were detected between the FB and ß-Lg emulsions in terms of oil droplet size which shown as volume weighted mean of oil droplet size D (4,3) and surface weighted mean diameter of oil droplet D (3,2), where the values of D (4,3) were 2.71 and 6.08 µm while, the values of D (3,2) were 1.93 and 3.34 µm for emulsions prepared with FB and ß-Lg proteins, respectively.

The large size of droplets in emulsion produced using FB protein may be related to its solubility. These results are interesting with the results in Fig. 1, also with stability of emulsion results in Fig. 3.

Stability of emulsions against creaming

The stability of emulsion is considered one of the main parameter in the food emulsion industry. It refers to the ability of an emulsion to prevent droplet creaming, coalescence, and flocculation. The emulsions, prepared with FB and ß-Lg proteins, were centrifuged at 3000 rpm to form serum layer. Then, the creaming stability of emulsions was studied as the turbidity of emulsions at 500 nm and the results are presented in Fig. 3. The results exposed that, at the whole time of the experiment ß-Lg emulsion was very stable compared to FB protein emulsion, where it did not exposure any creaming part till 65 min centrifugation. In contrast, the emulsion prepared using FB protein showed slowly decrease in the stability at the first 25 min followed by intensively decrease at the last 40 min centrifugation.

The low stability of emulsion which emulsified with FB protein may be related to low solubility of protein and the large oil droplets size, as mentioned above in Figs. 1 and 2. The low solubility of protein causes the decrease in the amount of protein at the oil –water interface, which is one of the most important reason for unstable emulsions, where a sufficient amount of protein is fixed to cover oil droplet completely to form more stable emulsion **(Aewsiri *et al.,* 2009)**.

Characterization of Faba Protein Hydrolysates

Degree of hydrolysis

When producing hydrolyzed protein, it is important to measure degree of hydrolysis (DH), using the change in protein content, and study the efficiency of protein hydrolysis reaction. Degree of FB protein hydrolysis was calculated for different hydrolysis times, starting from 0 min up to 180 min and the obtained results are illustrated in Fig. 4. The results indicated that with increasing the hydrolysis time from 0 min to the first 5 min, the hydrolysis of protein at both pH (1.5 and 3) was intensively increased. The degree of hydrolysis after about 10 min was not so much. Then, increasing the hydrolysis time for longer periods strongly affected the degree of hydrolysis**.** As shown also in Fig. 4, the degree of hydrolysis at pH 1.5 was higher than that at pH 3, where the values increased from 0% at 0 min to 87.3% after 180 min incubation at pH 1.5, while at pH 3 the values were increased from 0% to 67.4% when hydrolysis was run from 0 to 180 min. The same trend was observed during the hydrolysis of rapeseed protein isolates, whey protein, red tilapia fish protein and rice bran protein concentrate using alcalase, chymotrypsin, flavourzyme, neutrase thermolysin, protamex, and papain enzymes **(Chabanon *et al.,* 2007; Karamac and Rybarczyk, 2008; Dryakova´ *et al.,* 2010; Daud *et al.,* 2013; Wisuthiphaet *et al.,* 2015; Ahmadifard *et al.,* 2016).** Finally, it could be summarize that, the DH of protein is dependent on nature of the protein and the hydrolysis conditions, particular pH and time.

The change in the content of free amino groups

Fig. 5 represents the effect of hydrolysis time and pH on the content of free amino groups of FB protein. Results presented in the Figure clearly show that the percentage of free amino groups was increased for both pH with increasing the hydrolysis time. As seen from the obtained results, the percentage of free amino groups was 43.4% after first 5 min of hydrolysis at pH 3 and gradually increased by increasing hydrolysis time to reach 134.8% after 180 min of hydrolysis, while at pH 1.5 it was 30.5% after 5 min of hydrolysis and gradually

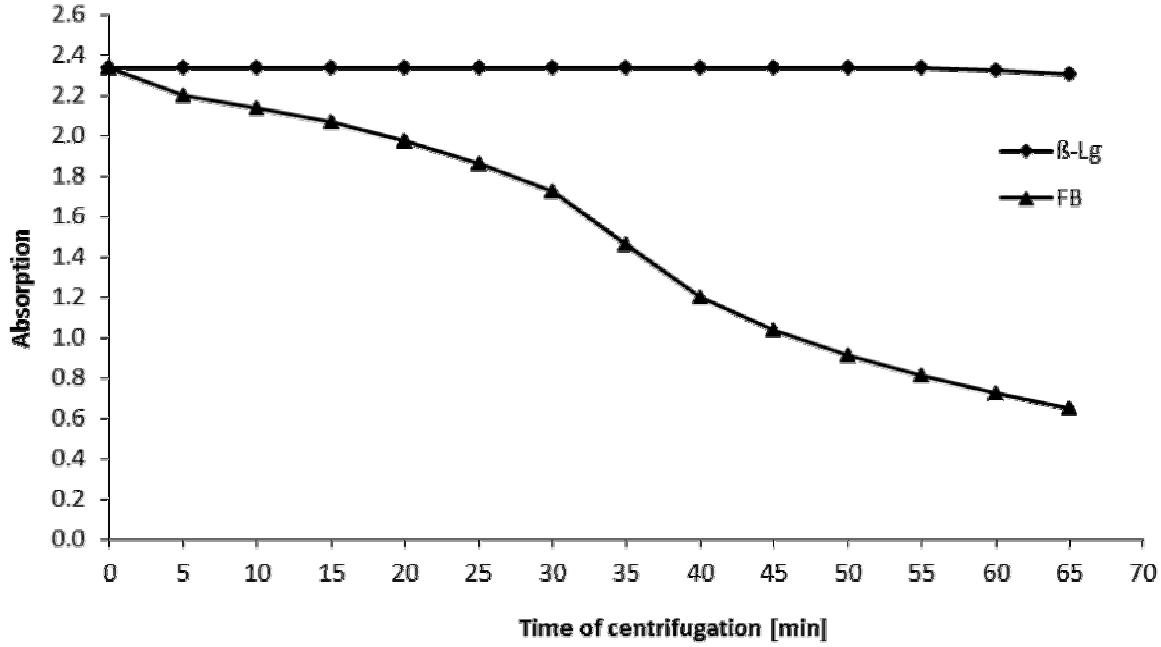

Fig. 3. Stability of emulsions prepared using faba and ß-Lg proteins against creaming

Where: ß-Lg, Beta-Lactoglobulin and FB, Faba bean proteins

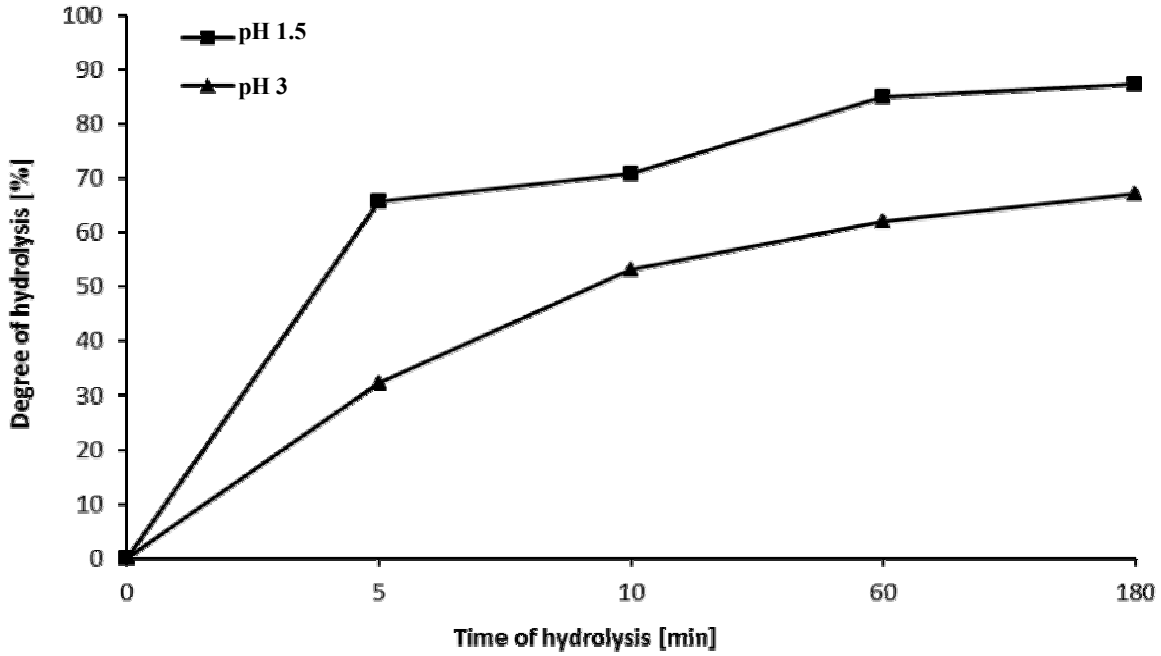

Fig. 4. Degree of hydrolysis (%) of faba bean protein with pepsin at two pH values for different times

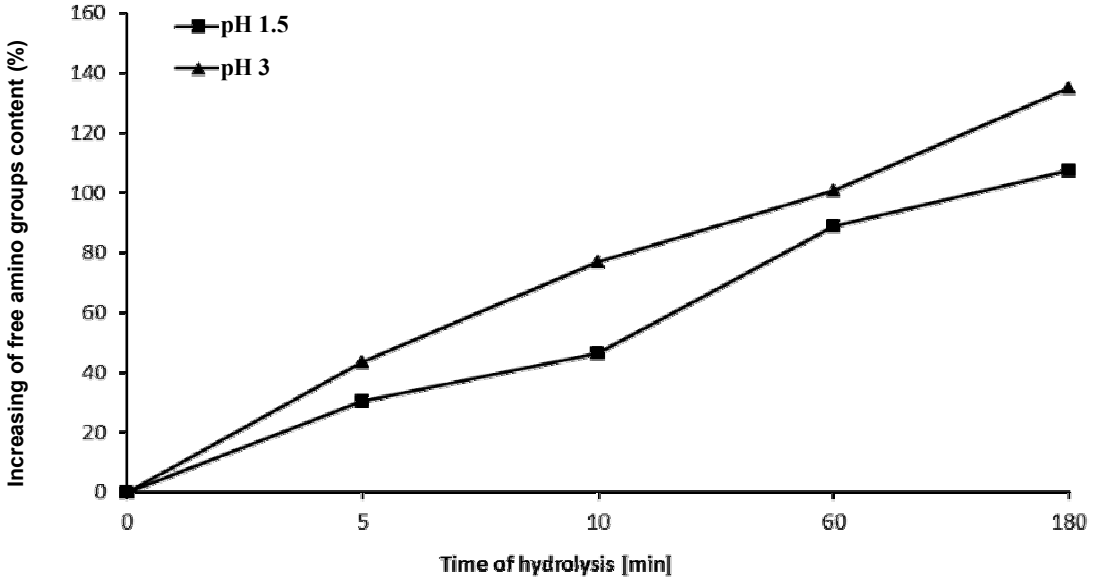

Fig. 5. Effect of enzymatic hydrolysis of faba bean protein at two pH values for different times on free amino groups content

increased to reach 107.4% after 180 min of hydrolysis. The results confirm that, the enzymatic hydrolysis of FB protein showed a positive relationship between the content of free amino groups and the hydrolysis time.

The change in protein content using RP-HPLC method

The change in native FB protein content, after incubation with pepsin at pH 1.5 and 3 for 0, 5, 10, 60, 180 min, was analyzed by RP-HPLC (Fig. 6). The results present in the Figure showed that the amount of protein was gradually decreased with increasing hydrolysis time. Hydrolysis at pH 1.5 exposed the highest decrease in area under curve where, the value was 3959744 mAU/s at 0 time and 847243 mAU/s after 180 min, ~ 4.7 fold. On the other hand, it recorded 1887093 mAU/s after incubation for 180 min at pH 3, ~ 2.1 fold.

Characterization of Molecular Weights of Hydrolysates

Molecular weight distribution using SDS-PAGE

The storage protein of FB belongs to 11S class proteins **(Ali *et al.,* 2012)**. To study the effect of pepsin on protein breakdown in FB protein, SDS-PAGE was performed on the hydrolysates after enzymatic hydrolysis at two different pH values (1.5 and 3) for 0, 5, 10, 60, and 180 min. The results are shown in Figs. 7A and B and Tables 2 and 3, where the line 1 in each figure shows the molecular weight of marker, line 2 the starting material, and lines 3 to 6 showed the hydrolysates obtained after incubation with pepsin at pH 1.5 and 3 for 5, 10, 60 and 180 min. SDS-PAGE separated extractable proteins into subunits and polypeptides. The SDS-electrophoretic profile of faba protein (Figs. 7A and B, line 2) contained 15 bands with molecular weight range between 11 and 98 KDa. According to **El-Saber (2010)**, the FB protein was separated using SDS-PAGE into 28 bands with a range of molecular weight range from 10 to 205 KDa, while **Hendawey and Younes (2013)** used the same technique to separate FB protein from different cultivars and they found around 32 bands with a range of molecular weight range from 14 to 95 KDa. As shown in Tables 2 and 3, the more intensive bands in FB protein at zero time are presented at molecular mass 98, 92, 71, 58, 47, 38, 34, 25, 22 and 21 KDa. These masses were identified as subunits of conglycinin, acidic and basic subunits of glycinin and lipoxygenase **(Barac *et al.,* 2006)** while, **Wu *et al.* (1998)** reported that the SDS–PAGE patterns of soya protein isolate contained five major bands; three of them were

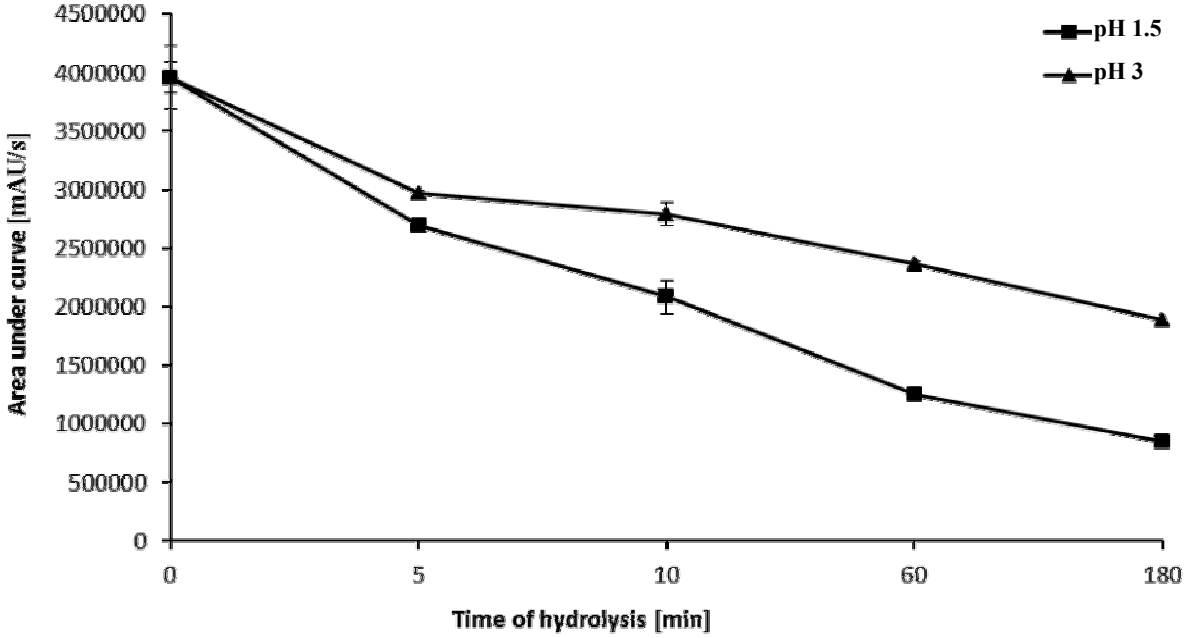

Fig. 6. Effect of enzymatic hydrolysis of faba bean protein at two pH values for different times on the content of protein using RP-HPLC

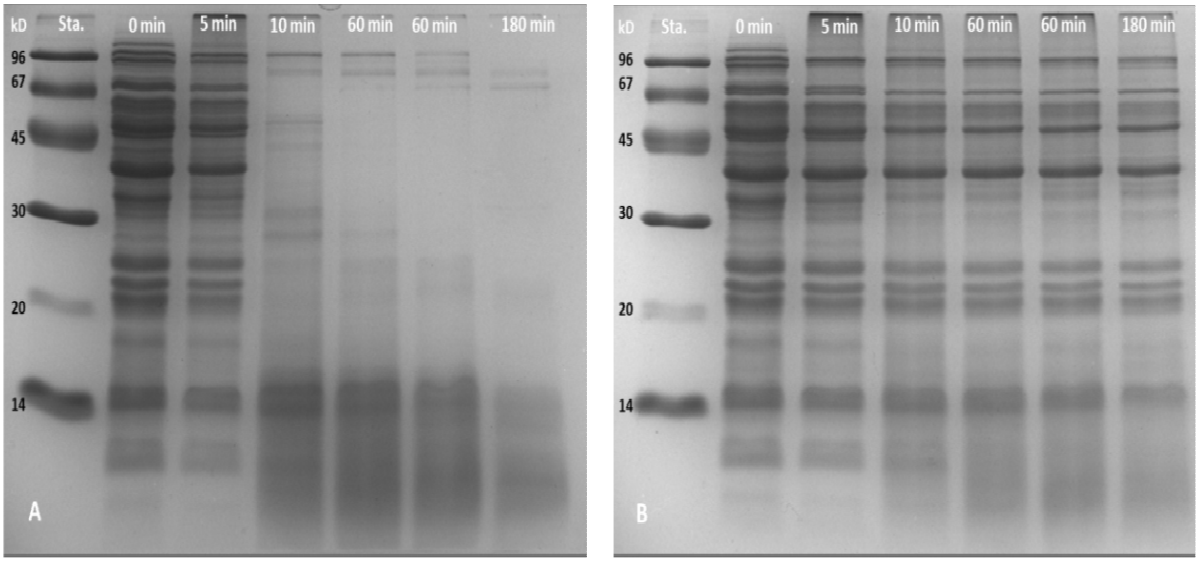

Fig. 7. SDS-PAGE profiles of faba bean protein after hydrolysis at pH 1.5 (A) and pH 3 (B) for different times. Sta, molecular weights of marker

Table 2. Molecular weights (KDa) and intensity of bands of faba bean protein after hydrolysis at pH 1.5 for different times

Mol. Wt. (KDa)	Intensity of bands				
	Time of hydrolysis				
	0 min	5 min	10 min	60 min	180 min
98	3065	2907	2282	1546	x
92	3103	2808	x	X	x
80	x	x	1861	1581	1361
71	3083	2822	x	X	x
69	x	x	x	1416	1474
58	3085	2875	x	X	x
47	3242	3076	x	X	x
38	3275	3094	x	X	x
34	3020	2695	x	X	x
32	2708	2423	x	X	x
25	2863	2552	1982	1404	x
22	2839	2541	x	1466	x
21	2866	2508	x	X	x
18	2427	2115	x	X	x
14	2792	2495	2733	2383	1919
12	2263	2057	2454	2325	1938
11	2299	2068	2493	2377	2077
10	x	x	2409	2457	2103
9	x	x	x	2246	2021

X means the band not detected

Table 3. Molecular weights (KDa) and intensity of bands of faba bean protein after hydrolysis at pH 3 for different times

Mol. Wt. (kDa)	Intensity of bands				
	Time of hydrolysis				
	0 min	5 min	10 min	60 min	180 min
98	2918	2870	2685	2491	2188
92	2915	2452	2355	X	1825
71	2850	2604	2380	2333	2222
58	2847	2711	2422	2297	2059
47	3050	2937	2675	2683	2605
38	3094	3009	2805	2756	2630
34	2820	2509	2086	1864	1804
32	2413	2145	1785	1583	1492
25	2574	2380	2066	1931	1838
22	2555	2366	2135	1958	1879
21	2575	2335	2186	2041	1918
18	1997	1750	1654	1505	1382
14	2420	2228	2215	2109	1849
12	1774	1665	x	X	x
11	1875	1713	x	X	x
10	x	x	1783	1845	1638
9	x	x	x	1766	1520

X means the band not detected

identified as 7S globulin, and two as 11S globulin, in addition to minor bands were identified as subunits of 11S globulin, and lipoxygenase. Results presented in Fig. 7A and illustrated in Table 2 clearly indicate that the intensity of bands produced at pH 1.5 was gradually decreased and many bands were disappeared. Moreover, some new bands represent as polypeptides were appeared compared to the hydrolysis at pH 3 which showed also decrease in the intensity of bands but only two bands were vanished (12 and 11 KDa) and some of new ones appeared (Fig. 7B and Table 3). FB protein showed many protein bands not completely digested by pepsin enzyme at pH 3 compared to at pH 1.5. The bands corresponding to non-hydrolyzed protein in FB were persisted till 180 min of hydrolysis at pH 3 while at pH 1.5 they were only persisted till 5 min, the bands exhibited until 5 min but then disappeared.

Molecular weight distribution using MALDI-TOF MS

The FB protein hydrolysates produced by pepsin at pH 1.5 and 3 were analysed with MALDI−TOF MS to identify the molecular weight of peptides (Figs. 8 A and B), which is an important parameter reflecting the proteins hydrolysis **(Li *et al.,* 2008)**. From the results of SDS-PAGE, the bands with molecular weight lower than 9 KDa cannot be identified so, these molecules were subject to MALDI-TOF MS. According to the literature, this is the first paper used this technique to identify the peptides, and the results are shown in Figs 8A and B. Results in these Figures showed the molecular weight of peptides between 500 and 4000 Da, where FB protein hydrolyzed at pH 1.5 for 5 min showed only one peptide with *m/z* 542 compared to two peptides with *m/z* 550 and 638 at pH 3. In general, results in Figures clearly indicate that FB protein was hydrolyzed well with 1% pepsin at pH 1.5 compared to pH 3, where the number of peptides was more, in addition the intensity of peaks was higher. On the other hand, pepsin incubated at pH 3 did not hydrolyze FB protein well even after 180 min of incubation. These results support the results of SDS-PAGE, degree of hydrolysis and RP-HPLC in the present study.

Antioxidant activity of hydrolysates by TEAC assay

The antioxidant activities of lyophilized hydrolysates of FB protein (μM TE/mg) were compared to each other and with that of native FB protein based on free radical scavenging capacity and the results are shown in Fig. 9. As shown in the Figure, hydrolysates by pepsin at pH 3 resulted in a slight higher antioxidant activity than hydrolysates by pepsin at pH 1.5. The antioxidant activity was higher at first 5 min then increased with increasing the time of hydrolysis. The value of antioxidant activity was 0.052 μM TE/mg protein for native protein, then the values increased notably with all tested hydrolysates, from 0.228 to 0.347 and from 0.213 to 0.337 μM TE/mg protein after incubation from 5 to 180 min at pH 3 and 1.5, respectively. As reported by **Graszkiewicz *et al.* (2007)** and **Lin *et al.* (2011)**, lysozyme and cystatin, and whole egg white proteins hydrolyzed with trypsin and alcalase showed a strong antioxidant activity. Also, hydrolysis of the whey protein, soya sauce lees, dairy protein concentrates, skim milk proteins and fish meat of red Tilapia with different enzymes increased the antioxidant activity **(Dryakova´ *et al.,* 2010; Yang *et al.,* 2011; Conway *et al.,* 2012; Daud *et al.,* 2013)**.

High degree of hydrolysis thus means high content of released amino groups and it should presumably show also good antioxidant activity in hydrolysates **(Dryakova´ *et al.,* 2010)**. Nevertheless, the antioxidant activity of hydrolysates was not found to be directly attributed to DH, suggesting that the activity is inherent to specific amino acid sequence. The results in the present work indicated that enzymatic hydrolysis using pepsin significantly ($p \leq 0.05$) improved the antioxidant potential of FB protein. A strong correlation between $ABTS^{+}$ scavenging activity and DH of pepsin hydrolysates of FB protein was observed. The proteins owe their antioxidant activity to their constituent amino acids such as aromatic, sulfur containing and basic amino acids which are capable to donate protons to free radicals **(Je *et al.,* 2005; Rajapakse *et al.,* 2005)**. It is possible that unfolding of the protein structure showing amino acids previously absent inside the native protein structure, causing an increase in the antioxidant activity so, it suggested that steric structure and molecular weight of peptides might exert more important role in scavenging DPPH radicals than the content of antioxidant amino acids **(Young *et al.,* 2011)**.

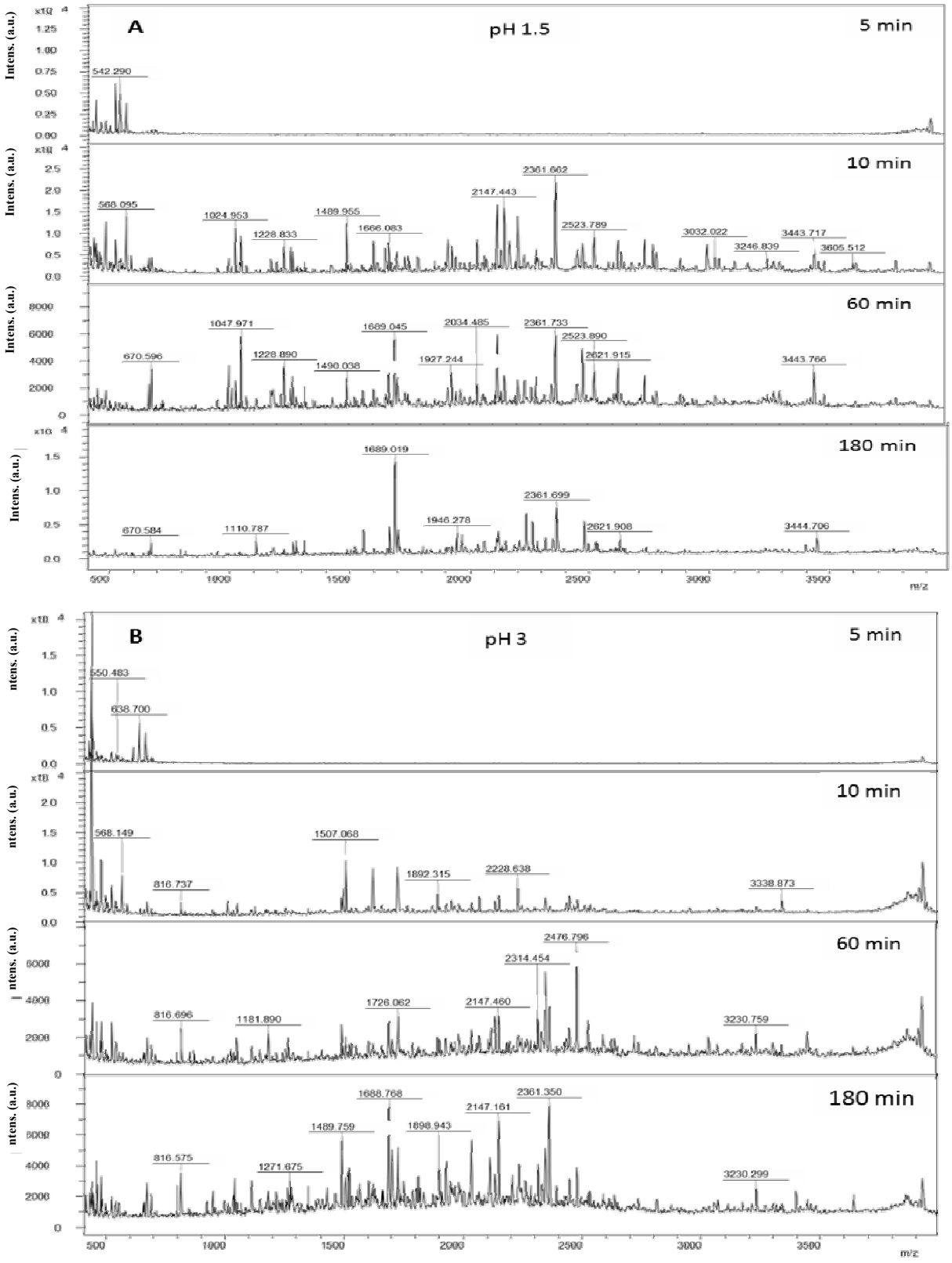

Fig. 8. Molecular weights (KDa) of faba bean protein hydrolysates after hydrolysis at pH 1.5 (A) and pH 3 (B) for different times

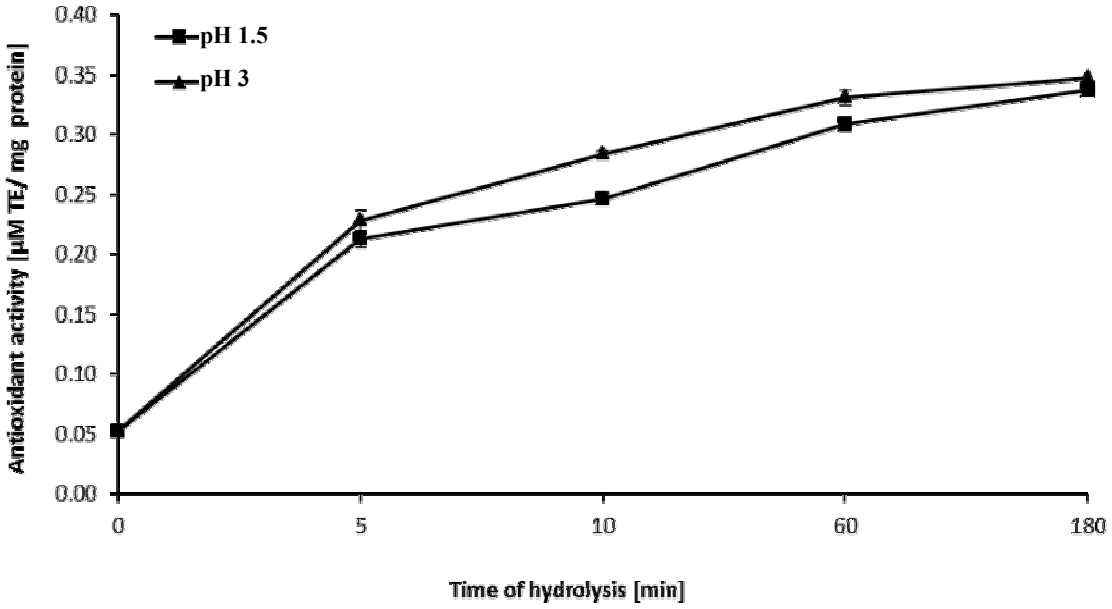

Fig. 9. Effect of enzymatic hydrolysis of faba bean protein at two pH values for different times on the antioxidant activity with TEAC assay

Conclusion

This study thus provides useful information for increasing the commercial value of faba bean protein as a multifunctional ingredient. It could be concluded that, the functional properties of native FB protein was lower than the properties of ß-Lg protein. These properties were improved using enzymatic hydrolysis process. FB protein could be hydrolyze by pepsin at pH 1.5. Hydrolysates produced at pH 1.5 were higher than which at pH 3. Also, pepsin may give higher antioxidant peptides after only 5 min incubation. Finally, test model systems closer to real food processing conditions would be helpful to evaluate whether plant protein hydrolysates could be a viable alternative for other functional protein sources. Moreover, the other functional properties of FB hydrolysates will be study in our future work.

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الخواص الوظيفية لبروتين الفول البلدى وتأثير التحليل الإنزيمي على نشاطه كمضاد للأكسدة

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تلعب البروتينات دورًا مهمًا في تحديد بنية وقوام المنتجات الغذائية المختلفة، لذا كانت أهداف هذا البحث هي دراسة الخصائص الوظيفية لبروتين الفول البلدى، صنف جيزة ٣، مقارنة ببروتين البيتالاكتوجلوبيولين (ß-Lg) ولتحليل هذا البروتين باستخدام أنزيم البيبسين عند مستويات pH (١,٥ و ٣) و فترات تحضين مختلفة (صفر ، ٥ ، ١٠ ، ٦٠ ، و ١٨٠ دقيقة)، بالإضافة إلى توصيف المتحللات الناتجة و فحص أنشطتها كمضادات للأكسدة، تم تقييم قابلية بروتين الفول للذوبان عند قيم pH مختلفة وكذلك خصائص استحلابة (الثبات ضد تكون الكريمة - حجم قطرات الزيت) مقارنة مع بروتين الـــ ß-Lg بيّنت النتائج أن قابلية الذوبان وخواص الاستحلاب لبروتين الفول كانت منخفضة جدًا مقارنةً ببروتين الـــß-Lg، وفيما يتعلق بعملية التحلل الإنزيمى، كانت درجة التحلل عند pH ١,٥ أعلى من درجة pH 3، أما بالنسبة لتوزيع الاوزان الجزيئية لمتحللات بروتين الفول فكانت في نطاق ٩– ٩٨ كيلو دالتون عند استخدام طريقة SDS-PAGE بينما كانت الببتيدات في المدى من ٥٠٠ - ٤٠٠٠ دالتون باستخدام طريقة MALDI-TOF MS حيث أكدت نتائج كلتا الطريقتين أن التحلل الانزيمى عند درجة pH 1.5 كان أعلى من pH 3، وعلاوة على ذلك، أدى التحلل الأنزيمي إلى تحسن كبير في نشاط بروتين الفول كمضاد للأكسدة، وأظهرت النتائج أن المتحللات المنتجة في درجة pH 3 أعطت نشاط مضاد للأكسدة أعلى قليلا من المنتجة عند استخدام pH 1.5 في جميع فترات التحضين، وأخيراً، تشير هذه النتائج إلى أنه يمكن استخدام متحللات بروتين الفول في إعداد الأغذية الوظيفية وكمضادات أكسدة طبيعية لمنع عملية الأكسدة في المنتجات الغذائية.

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