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Physicochemical and antioxidative characteristics of black bean protein hydrolysates obtained from different enzymes

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ABSTRACT

Black bean is an excellent protein source for preparing hydrolysates, which attract much attention due to their biological activity. The objective of this study was to characterize the physicochemical and antioxidant properties of black bean protein, hydrolyzed by ficin, bromelain or alcalase until 300 min of hydrolysis. Results showed that bromelain and alcalase hydrolysates possessed higher degree of hydrolysis (DH) than that of ficin, thereby presenting different ultraviolet absorption, fluorescence intensity and circular dichroism. Moreover, all hydrolysates possessed the capacity to scavenge DPPH radical with the lowest IC_{50} of 21.11 μg/mL, as well as to chelate ferrous ion (Fe^{2+}) with the IC_{50} values ranging from 6.82 to 30.68 μg/mL. Intriguingly, the oxidation of linoleic acid, sunflower oil and sunflower oil-in-water emulsion was remarkedly retarded by the three selected protein hydrolysates, especially by bromelain-treated protein hydrolysate, which might attribute to their high hydrophobicity and emulsifying properties. These findings can provide strong support for black bean protein hydrolysates to be employed in food products acting as natural antioxidant alternatives.

1. Introduction

Pulses contain abundant amounts of edible proteins (20–40%), which are considered as excellent ingredients both economically and nutritionally (Kudre, Benjakul, & Kishimura, 2013; Ustimenko-Bakumovsky, 1983). As a representative pulse crop, protein content of black bean (*Phaseolus vulgaris L.*) is higher than that of soybean and even that of milk, meat or eggs. However, about half of total seed proteins in beans account for their low digestibility (Rui, Boye, Simpson, & Prasher, 2012). The presence of antinutritional compounds also restricts the nutritional value of pulse protein (López-Barrios, Gutiérrez-Uribe, & Serna-Saldívar, 2014).

Enzymatic hydrolysis is an attractive approach for utilizing black bean protein to achieve more value out of what has been recognized. Intriguingly, enzymatic hydrolysis has many merits, such as mild reaction conditions, high product yields, and low energy cost (Woiciechowski et al., 2014; Zheng, Wei, et al., 2018). Recently, so much attention has been paid to explore the influences of selective enzymatic hydrolysis on the physicochemical and functional properties of pulse proteins (Betancur-Ancona, Sosa-Espinoza, Ruiz-Ruiz, Segura-Campos, & Chel-Guerrero, 2013; Carrasco-Castilla et al., 2012; Wani, Sogi, Shivhare, & Gill, 2015; Xie, Du, Shen, Wu, & Lin, 2019). Specifically, proteolysis is capable to convert proteins into peptides with desired size, charge and surface hydrophobic properties, and further to improve emulsifying properties (Evangelho et al., 2017; Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010). Moreover, enzymatic hydrolysis has the favorable potential to remove antinutritive factors (Moure, Sineiro, Domínguez, & Parajó, 2006).

In addition to the physicochemical properties, enzymatic hydrolysis of pulse proteins has been extensively studied for the potential bioactive capacity, especially for antioxidant activity. For instance, Evangelho et al. (2017) investigated the antioxidant activity of black bean protein hydrolysates obtained from pepsin and alcalase digestion. Similarly, Yust, Millán-Linares, Alcaide-Hidalgo, Millán, and Pedroche (2012) found chickpea protein after sequential treatment with alcalase and flavourzyme had stronger antioxidant activity than chickpea protein isolate. Generally speaking, protein hydrolysates are prevailingly evidenced in scavenging free radicals and chelating metal ion, thereby confirming their antioxidant capacity (Chi, Wang, Wang, Zhang, & Deng, 2015; Jang, Liceaga, & Yoon, 2016; Ohata, Uchida, Zhou, & Arihara, 2016; Sabeena Farvin et al., 2016). To our knowledge, certain metal ions and free radicals are implicated in lipid peroxidation, which imparts undesirable off-flavors and potential hazards to food products (Pihlanto, 2006). Consequently, protein hydrolysates with antioxidant

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activity should have the potential to inhibit lipid peroxidation, and further prolong the shelf-life of food products.

It is well-acknowledged that the physicochemical and antioxidant properties of protein hydrolysates mainly depend on enzyme type and hydrolysis process, particularly hydrolysis time. However, there is limited research regarding the impacts of different enzyme modification on the physicochemical and antioxidant properties of black bean protein. Therefore, the objective of the present work was to characterize the effects of proteases employed and hydrolysis time on the physicochemical and antioxidative properties of protein hydrolysates derived from black bean. Based on hydrophobicity and antioxidant activity, we selected three hydrolysates prepared by ficin, bromelain and alcalase to evaluate whether these have the potential to retard the lipid oxidation of linoleic acid, sunflower oil and oil-in-water emulsion.

2. Materials and methods

2.1. Materials

The black bean was obtained from Shiyuedaotian Company (Beijing, China). Ficin and bromelain was purchase from J&K scientific Co., China, and alcalase was kindly donated by Novo Co., Denmark. Sunflower oil was purchased from local supermarket in China. 1,1-Diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) and ferrozine were purchased from Sigma-Aldrich. Dithiothreitol (DTT), linoleic acid and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Macklin Co. Ltd. (Shanghai, China). All other chemicals used were of analytical reagent grade.

2.2. Protein extraction

Black bean was ground in blender and passed through an 80-mesh sieve. Bean flour was defatted twice by using four volumes of hexane, and then air-dried overnight. According to the method of alkaline extraction and acid precipitation, the defatted bean flour was suspended in deionized water (1:8, w/v) and adjusted to pH 8.0 with 15 mM NaOH. The resulting slurry was stirred for 60 min at room temperature and centrifuged for 10 min at 10,000 × *g*. The supernatant was collected and adjusted to pH 4.5 with 2.0 M HCl, and then centrifuged at 10,000 × *g* for precipitation. The precipitated protein was resuspended into deionized water and adjusted to pH 7.0. Finally, the protein solution was stored at − 80 °C and lyophilized, and its protein content was 82.52 ± 0.78%.

2.3. Enzymatic hydrolysis of black bean protein

Black bean protein was first treated at 100 °C for 20 min in an experimental short-wave infrared radiation equipment (Senttech Infrared Technology Co., Ltd, Taizhou, China). Then the sample was dissolved in deionized water to obtain a 10% solution (w/v). The hydrolysis with ficin (enzyme/substrate ratio, 2%) was conducted at 65 °C and pH 5.7, bromelain treatment (enzyme/substrate ratio, 2%) was conducted at 55 °C and pH 7.0, while hydrolysis with alcalase (enzyme/substrate ratio, 2%) was performed at 55 °C and pH 8.5. The hydrolysates were collected at time intervals of 1, 2, 3, 4, and 5 h, and the aliquots were heated at 90 °C for 15 min to inactivate enzymes. After cooling down to the room temperature, the pH of sample was adjusted to neutral. The solutions were centrifuged at 10,000 × *g* for 15 min, and the supernatants were collected for further study.

2.4. Degree of hydrolysis (DH)

DH of black bean protein was determined by an o-phthaldialdehyde (OPA) method (Nielsen, Petersen, & Dambmann, 2001). Briefly, 200 μL sample (or control) was mixed with 1.5 mL OPA reagent and incubated for exactly 2 min at room temperature. Then the absorbance of the mixture was measured at 340 nm in a UV–visible spectrophotometer (Ultrospec 7000, GE Healthcare, USA).

2.5. SDS-PAGE electrophoresis

The electrophoretic profile of protein and hydrolysate was performed as described in our previous report (Jiang, Zhang, Zhao, & Liu, 2018). Briefly, protein sample was mixed with SDS-PAGE sample loading buffer (Beyotime, Shanghai, China) at the ratio of 4:1. The mixture was heated at 100 °C for 10 min. After cooling down to room temperature, 10 μL solution was loaded into the gels (4% stacking gel and 16% separating gel). Tricine SDS-PAGE was conducted in a Mini-PROTEAN Tetra Cell system (Bio-Rad, USA). Protein ladder of 10–200 kDa (Beyotime, Shanghai, China) was used as molecular weight marker and gels were stained using Coomassie blue.

2.6. Structural characterization of black bean protein hydrolysates

2.6.1. UV absorption spectroscopy

As described by Du et al. (2018), UV-spectrum of the hydrolysate was determined after appropriate dilution using UV–visible spectrophotometer (GE Healthcare, USA) in the range of 200–800 nm. All samples were prepared and determined at room temperature.

2.6.2. Relative fluorescence intensity

The relative fluorescence of protein hydrolysate was performed using the method described by J. A. D. Evangelho et al. (2017) with slight modification. Briefly, 1.5 mL of protein hydrolysates (1 μg/mL) in 10 mM phosphate buffer (pH 7.0) were added to falcon tubes containing 20 μL of solution of ANS stock solution (8 mM). The fluorescence intensity of mixture was determined in the 400–600 nm region with a spectrofluorometer (F-7000, Hitachi, Japan).

2.6.3. Circular dichroism (CD) spectroscopy

CD spectroscopy was used to observe the structure changes of hydrolysates by the method of Mohammadian and Madadlou (2016). Wavelengths between 190 and 260 nm were scanned for protein hydrolysate diluted in pure water by using a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd., UK) with a path length of 1 mm.

2.7. Emulsifying properties

The method employed by Suppavorasatit, De Mejia, and Cadwallader (2011) was used to evaluate emulsifying activity index (EAI) and emulsion stability index (ESI) of protein samples. Briefly, dispersions containing 3 mL of 0.2% hydrolysates were homogenized with 1 mL corn oil at 20,000 rpm for 1 min using a homogenizer (IKA T25, Staufen, Germany). A 25 μL aliquot of emulsion was immediately diluted with 2.5 mL of 0.1% (w/v) SDS solution and also for 1 h after homogenization. The absorbance of mixture was measured at 500 nm with a UV–visible spectrophotometer. Also, the microstructure of emulsion was observed using a polarized light microscope (Leica, Germany) installed with a Leica DFC450 video camera.

2.8. Antioxidant activity of black bean protein hydrolysates

2.8.1. DPPH radical scavenging activity

DPPH radical scavenging activity of protein hydrolysate was determined as described by Zheng, Si, Ahmad, Li, and Zhang (2018) with a slight modification. Briefly, two-fold serial dilutions of protein hydrolysates were prepared, and 50 μL aliquot was mixed with 50 μL of 95% ethanol containing 0.1 mM DPPH. The mixture was allowed to stand in the dark for 30 min, and the absorbance was monitored at 517 nm with a microplate reader (SpectraMax 190, Molecular Devices, USA).

2.8.2. Metal-chelating activity

The ability to chelate the ferrous ion (Fe^{2+}) was determined according to the reported method (Taheri, Sabeena Farvin, Jacobsen, & Baron, 2014) with minor modification. 50 μL of two-fold serial dilutions of protein hydrolysates was mixed with 100 μL of 20 μM $FeCl_2$ in the 96-well plates. The mixture was then reacted with 100 μL of 0.5 mM ferrozine for 10 min at ambient temperature. Deionized water instead of sample was control. Absorbance of the resulting solution was measured at 562 nm.

2.8.3. Antioxidant activity of hydrolysates in linoleic acid

The antioxidative effects of protein hydrolysates in a linoleic acid model system were evaluated by ferric thiocyanate method (Osawa & Namiki, 1981). Briefly, 1 mL protein sample (50 μg/mL) and 2 mL phosphate buffer (0.05 M, pH 7.0) was added to 2.5% linoleic acid in ethanol (1 mL) and kept in screw cap containers under dark condition at 60 °C. The reaction solution (5 μL) was added into 96-well plate, following by 235 μL of 75% ethanol, 5 μL of 30% (w/v) ammonium thiocyanate, and 5 μL of 0.02 M ferrous chloride solution (3.5% HCl). After 3 min incubation, the absorbance of color solution was measured at 500 nm in a microplate reader. The ferric thiocyanate values were evaluated every day for 7 days.

2.8.4. Antioxidant activity of hydrolysates in sunflower oil

Antioxidant activity of black bean hydrolysates on the oxidation stability of sunflower oil was detected by means of rancimat test, using an 892 Professional Rancimat Instrument (Metrohm Ltd., Herisau, Switzerland). Sunflower oil (4 g) was mixed with 0.5% sample in glass test tube and treated for 60 min with ultrasound. Then the induction time of sunflower oil with or without the addition of hydrolysate was detected under constant airflow of 20 L/h within the thermostat-controlled block heater at 120 ± 1.6 °C. The experiments were carried out at least in duplicate.

2.8.5. Antioxidant activity of hydrolysates in oil-in-water emulsion

The oil-in-water (O/W) emulsion was prepared with sunflower oil due to its low oxidative stability. 30% sunflower oil, 1% Tween 20, and ultrapure water were stirred for 30 min at room temperature. The mixture was then homogenized in a homogenizer (IKA T25) (12,000 rpm/2 min), followed by a two-stage high-pressure homogenizer (150/50 bar) with an AH-2010 homogenizer (ATS Engineering Inc., Ontario, Canada). The size of droplets, measured by NanoBrook Omni (Brookhaven Instruments, New York, USA), was 1.51 ± 0.16 μm. Subsequently, emulsions were prepared by addition of hydrolysate samples to obtain the protein concentration of 400 μg/mL. Emulsion without addition was the control. The malonaldehyde (MDA) production in emulsion during 8 days storage was determined by TBARS test (Caetano-Silva, Mariutti, Bragagnolo, Pacheco, & Netto, 2018). Briefly, 1.5 mL emulsions were transferred to test tubes. 1 mL of 1% thiobarbituric acid (w/v) and 2.5 mL of 10% trichloroacetic acid (w/v) were added to the test tubes, mixed and incubated for 30 min in a boiling water bath. After incubation, the mixture was cooled down to room temperature in an ice bath. Aliquots of 2.5 mL were mixed with equivalent trichloromethane and centrifuged at 3000 × *g* for 10 min. The absorbance of supernatants was measured at 532 nm. Besides, the microstructure of stored emulsion was analyzed by optical microscopy. Observations were carried out at ambient temperature unless otherwise stated.

2.9. Statistical analysis

All results were expressed as mean values with indication of standard deviation. One-way analysis of variance (ANOVA) combined with Student's *t*-test were used to determine the differences among treatments ($P < 0.05$) by Graphpad prism 7 (Graphpad Software Inc., San Diego, USA). The concentration of hydrolysate fraction that exhibited 50% of antioxidant activity (IC_{50} value) was also calculated by Graphpad prism 7. The resulting data were investigated graphically using OriginPro 2016 (OriginLab, Northampton, MA).

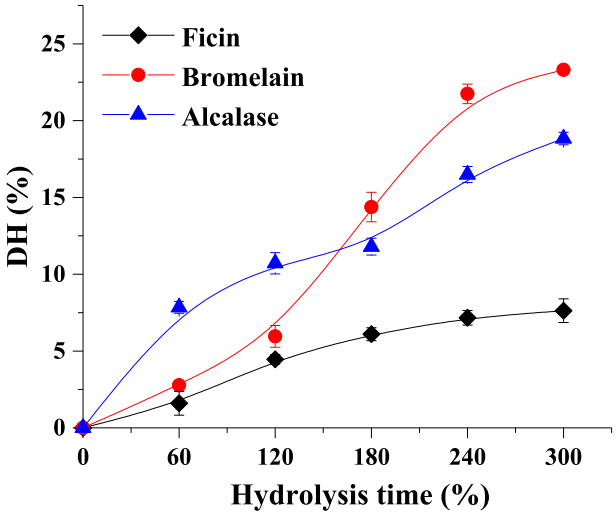

Fig. 1. Hydrolysis curves of black bean protein hydrolyzed by ficin, bromelain and alcalase.

3. Results and discussion

3.1. Degree of hydrolysis (DH)

DH is an important parameter to measure the hydrolysis of protein, which can determine the functional properties and biological activities of protein hydrolysate (Adler-Nissen, 1986; Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Arun, 2010). Herein, ficin, bromelain or alcalase was incubated with black bean protein under different hydrolysis time to evaluate the DH as presented in Fig. 1. Not surprising, DH values obtained from all these three proteases increased with increasing the hydrolysis time. Alcalase promoted a significant growth ($P < 0.05$) in the DH during the first 2 h of reaction, while bromelain achieved an obvious increase after 120 min. At the end of reaction time, the DH by bromelain and alcalase were 23.31 ± 0.19% and 18.84 ± 0.41%, respectively. Compared with bromelain and alcalase, ficin displayed the lowest rate of hydrolysis on the black bean protein. At the hydrolysis time of 4 h, ficin-treated protein hydrolysate achieved the maximum DH of 7.63 ± 0.77%.

3.2. SDS-PAGE electrophoresis

The hydrolysis of black bean protein using ficin, bromelain or alcalase was also monitored by SDS-PAGE. As described in Fig. 2A, the black bean proteins presented the predominant band between 60 and 85 kDa, possibly coinciding with legumin (69 kDa) or other unnamed 11S proteins (J. A. Evangelho et al., 2017; Kudre et al., 2013). Another intense band was observed at 30–50 kDa, which most likely corresponded to 7S fractions of seed storage protein (e.g. phaseolin) or probably to lectin, arcelin and phytohemagglutinin (Mojica & Mejía, 2015). As for the hydrolysate obtained from ficin degradation, the intensity of the higher molecular weight bands decreased, and lower molecular weight bands gradually increased as a function of hydrolysis time. Furthermore, protein bands corresponding to molecular mass higher than 40 kDa observed in black bean proteins at least partially disappeared in the ficin-treated hydrolysate. Similarly, the bromelain-treated hydrolysates showed the explicit and uniform bands below 40 kDa, regardless of hydrolysis time (Fig. 2B). As depicted in Fig. 2C, the obvious shifts from higher molecular weight bands to lower molecular weight bands were observed in alcalase-treated hydrolysates up to

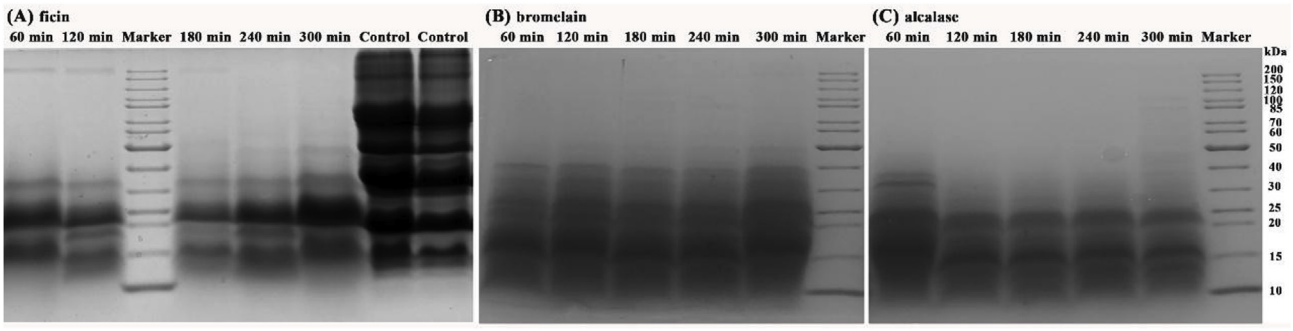

Fig. 2. Analysis by SDS-PAGE of black bean protein and hydrolysates obtained by ficin (A), bromelain (B) and alcalase (C). Control: untreated black bean protein; 60–300 min of enzymatic hydrolysis, respectively; Marker: molecular weight markers from 10 to 200 kDa.

240 min of hydrolysis, confirming the effectiveness of enzymatic treatments. However, the electrophoretic patterns of protein hydrolysates treated with alcalase for 5 h showed the presence of several bands larger than 40 kDa. This phenomenon may ascribe to the aggregation of some peptides occurred by hydrogen bonds, which is consistent with the findings of Evangelho et al. (2017) and Rui, Boye, Ribereau, Simpson, and Prasher (2011). Overall, all band patterns for these hydrolysates indicated the marked downward shifts to lower molecular mass units, implying their potential nutritive values (Vijayalakshmi, Lemieux, & Amiot, 1986; Zheng et al., 2014).

3.3. Structural characterization of black bean protein hydrolysates

3.3.1. Ultraviolet analysis

Fig. 3 A-C showed the ultraviolet–visible spectra of black bean hydrolysates obtained with ficin, bromelain and alcalase. Obviously, enzymatic hydrolysis caused the pronounced hypsochromic shift of the ultraviolet absorption bands in comparison with the control group. The shift probably was caused by the exposure of aromatic chromophores and the conformational changes of protein after enzymatic treatment (Donovan, 1969; Schmid, 2001; Zhao, Xiong, & McNear, 2013). All three hydrolysates had the similar ultraviolet–visible shapes over the wavelength range of 200–800 nm. However, the difference between the ultraviolet area and visible area absorption spectra of each hydrolysate was apparent. The maximum absorption peak at around 210 nm was observed for the hydrolysates, characterizing the double bonds or triple bonds (Nie, Xie, Fu, Wan, & Yan, 2008). A slight absorption shoulder appeared at 280 nm, implying the cleavage of black bean protein under the action of enzymes.

3.3.2. Relative fluorescence intensity

The fluorescence emission spectra of ANS with binding to hydrolysates were recorded to describe the conformational changes of black bean protein as a function of enzymatic hydrolysis (Fig. 3D–F). It is well acknowledged that ANS prefers to bind the exposed hydrophobic regions in partially unfolded proteins, indicating the positive correlation between hydrophobicity and relative fluorescence intensity (Kronman & Holmes, 2010; Pallarès, Vendrell, Avilés, & Ventura, 2004). As depicted in Fig. 3D, the highest fluorescence emission spectra were resulted from the hydrolysates treated with ficin for 240 and 300 min, coinciding with the 2 h hydrolysis of black bean protein. Interestingly, the hydrolysis of black bean protein with ficin for 3 h led to a much lower fluorescence intensity than these for control and other hydrolysates. This probably due to re-bury the hydrophobic amino acids (e.g. tyrosine, phenylalanine and tryptophan) inside the protein cluster, while the exposure of buried hydrophobic groups was promoted as the increment of hydrolysis time (Evangelho et al., 2017). Unlikely, the almost overlapping fluorescence emission spectra were observed in all bromelain-treated protein hydrolysates, excluding that after 60 min of hydrolysis (Fig. 3E). It was worth noting that relative fluorescence intensity of all the hydrolysates obtained from bromelain was superior to that of control, demonstrating the acceerative effects of bromelain hydrolysis on the exposure of hydrophobic residues. As for alcalase hydrolysis, different hydrolysis time led to various fluctuations of the fluorescence, demonstrating the hydrophobicity of hydrolysate was partially determined by the hydrolysis time (Fig. 3F). Specifically, the maximum and minimum fluorescence emission spectra were obtained from the hydrolysates treated with alcalase for 240 and 180 min, respectively, corresponding to those of ficin hydrolysates.

3.3.3. Circular dichroism (CD)

CD spectroscopy has been increasingly recognized as a favorite and valuable technique for evaluating the changes in both the secondary and tertiary structure of proteins (Cabra et al., 2005; Ren, Ma, Mao, & Zhou, 2014; Whitmore & Wallace, 2010). Given the changes observed in molecular weight profile of black bean hydrolysates, we reckoned that their structure would also change. Therefore, we investigated the influence of enzymatic hydrolysis on structural changes of black bean proteins by means of CD spectroscopy. As shown in Fig. 3G, the CD spectrum of black bean protein without proteolysis showed a strong positive peak at 192 nm and a dominant negative peak at 208 nm, implying the α-helix structure. Ficin hydrolysis could cause significant shift of the negative bands and remarkable decrease of ellipticity in the 190–200 nm region, indicating the disruption of ordered structures. CD spectra of ficin hydrolysate after 60, 180, and 300 min hydrolysis mainly existed under the random coil conformation, while the remaining hydrolysates tended to emerge β-sheet conformation. Similarly, obvious blue shifts of the negative peaks from 208 nm to 200–204 nm were observed in all bromelain-treated hydrolysates, except in the hydrolysate after 300 min treatment (Fig. 3H). Interestingly, the 300 min-bromelain-treated hydrolysate presented a remarkable red shift of spectrum and declination of ellipticity in comparison with black bean protein (control). Alcalase treatment tended to induce an increase in β-sheet and random coil, and a decrease in α-helix of black bean protein (Fig. 3I). The loss of α-helix was also found in the enzymatic hydrolysis of soybean protein (Achouri & Wang, 2001; Zhao et al., 2013). A random coil conformation was prevailingly adopted within the hydrolysate after 240 min hydrolysis, whereas secondary structure of 5 h-alcalase-treated hydrolysate contained α-helix. The aggregation ability of protein hydrolysate obtained after 300 min alcalase hydrolysis might account for such conformation behavior, which was consistent with the aforementioned SDS-PAGE results.

3.4. Emulsifying properties

The emulsifying activity index (EAI) of black bean hydrolysates under different hydrolysis time were presented in Fig. 4A. EAI of ficin hydrolysate showed in a time-dependent manner, while its emulsifying activity was much lower than that of bromelain hydrolysate and alcalase hydrolysate. Unlikely, bromelain-treated hydrolysate achieved the

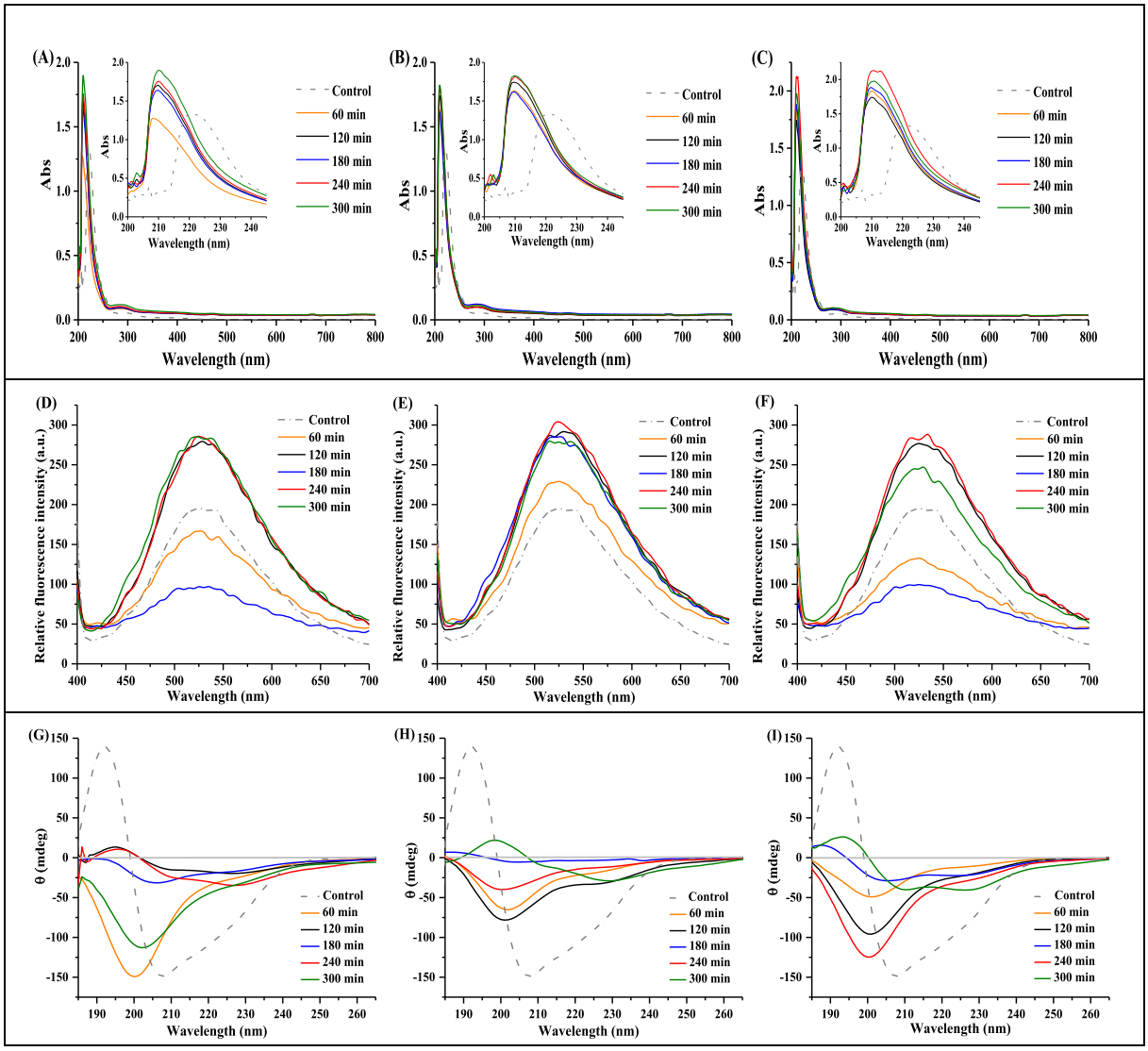

Fig. 3. Structural characterization of black bean protein and hydrolysates. UV–visible spectra of black bean protein and hydrolysates prepared with ficin (A), bromelain (B) and alcalase (C); Relative fluorescence spectra of black bean protein and hydrolysates prepared with ficin (D), bromelain (E) and alcalase (F); Circular dichroism (CD) spectra of black bean protein and hydrolysates prepared with ficin (G), bromelain (H) and alcalase (I).

lowest EAI at the hydrolysis time of 2 h, after which the emulsifying activity was enhanced with further increases in time. Alcalase-treated hydrolysate reached the highest EAI at the last moment of investigated time. The excellent emulsifying activity of all hydrolysates obtained by 5 h hydrolysis might due to the presence of hydrophobic residues and small peptides, which may diffuse rapidly and be adsorbed instantaneously to the surface of freshly formed oil droplets during homogenization (Giménez, Alemán, Montero, & Gómez-Guillén, 2009; Zheng, Wei, et al., 2018).

Emulsion stability index (ESI) measures the capacity of an emulsion to resist breakdown (Wu, Hettiarachchy, & Qi, 1998). ESI of black bean protein hydrolysates under different hydrolysis time was showed in Fig. 4B. Both ficin hydrolysate and bromelain hydrolysate presented similar stabilization behavior on the emulsion as a function of time. The highest ESI (983.94 ± 50.12 min) was achieved at 60 min-ficin-treated hydrolysate, while hydrolysate obtained by bromelain hydrolysis for 4 h had excellent emulsion stability (634.07 ± 29.89 min). Alcalase-treated hydrolysates exhibited the emulsion stability in the range from 138.37 ± 0.51 to 821.4 ± 0.00 min. On the contrary with EAI, these three protein hydrolysates after 5 h treatment displayed less efficient in stabilizing emulsions, probably attributing to desorption of small peptide from the interface post homogenization (Adjonu, Doran, Torley, & Agboola, 2014). Overall, ESI of all the hydrolysates ranged between 138.37 ± 0.51 and 983.94 ± 50.12 min, which were much higher than those hydrolysates from herring brine protein (Taheri et al., 2014), chickpea protein (Ghribi et al., 2015) and wheat gluten (He, Yang, & Zhao, 2019).

It is well-acknowledged that emulsion stability associates strongly with the droplet size, which may be influenced by protein emulsifier employed. As shown in Fig. 4C, the microstructure of emulsion varied with the hydrolysate obtained by different proteases and hydrolysis time. Specifically, the emulsions prepared with hydrolysates under ficin treatment of 120 or 180 min had larger droplets than others, and obvious flocculated particles were observed in the emulsion with 240 min-ficin-treated hydrolysates. For emulsion prepared with bromelain hydrolysate, some oil droplets were in their individual form, while other oil droplets in emulsion tended to cluster and flocculate as the increasing hydrolysis time of black bean protein. The emulsions prepared from alcalase presented larger and denser droplets in comparison with those emulsions with bromelain hydrolysates. Interestingly, emulsions with higher ESI had smaller and less flocculated droplets, corresponding to the view that formation of small droplets in the emulsions

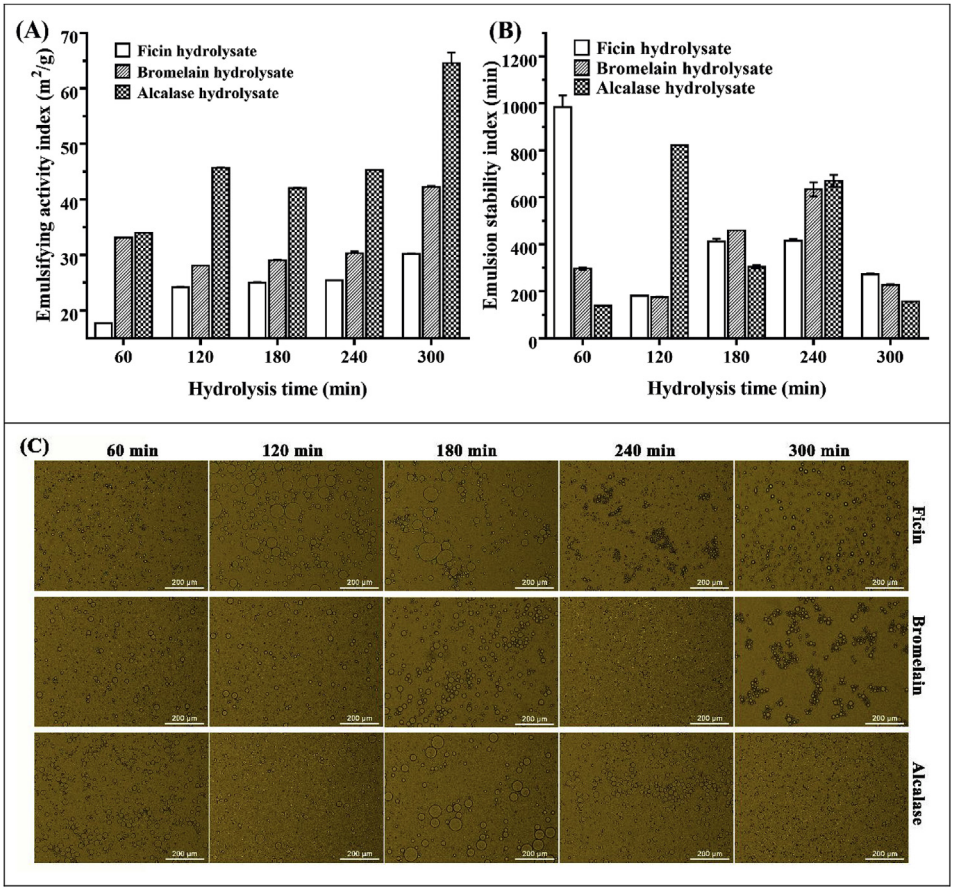

Fig. 4. Emulsifying characterization of black bean protein hydrolysates obtained by ficin, bromelain and alcalase treatment. (A) Emulsifying activity index (EAI, m^2/g) of black bean protein hydrolysate; (B) Emulsion stability index (ESI, min) of black bean protein hydrolysate; (C) Light micrographs of the prepared emulsions.

and lower coalescence of droplets were beneficial for the emulsifying properties and emulsion stability of emulsions (Evangelho et al., 2017; Kimura et al., 2008).

3.5. Antioxidant activity of black bean hydrolysate

3.5.1. DPPH radical scavenging activity

As a stable free radical, DPPH has been widely used as an indicator to investigate the scavenging activity of antioxidants (Jang et al., 2016; Klompong, Benjakul, Kantachote, & Shahidi, 2007; Zheng, Si, et al., 2018). Thus, the DPPH radical scavenging activity of black bean hydrolysates was detected and presented in Fig. 5A–C. DPPH scavenging activity of each hydrolysate presented in a dose-dependent manner until the radicals were wiped out. Hydrolysis was beneficial to elevate the DPPH radical scavenging activity of black bean hydrolysate. Specifically, black bean protein after 4 h ficin hydrolysis achieved the highest DPPH radical scavenging activity, with the IC_{50} value of 24.24 μg/mL (Fig. 5A). Compared with ficin hydrolysate, the bromelain hydrolysate under the same condition exhibited lower IC_{50} value (21.23 μg/mL), indicating its superior capacity to scavenge DPPH radicals (Fig. 5B). This might be relevant to the precedence of bromelain in the hydrolysis of black bean protein over ficin, as mentioned the results of DH. Unlike the hydrolysates produced by plant proteases, alcalase-treated hydrolysate achieved its strongest DPPH radical scavenging activity (IC_{50} = 21.11 μg/mL) at the hydrolysis time of 2 h (Fig. 5C). Interestingly, the hydrolysates with the strongest DPPH radical scavenging activities were produced under ficin, bromelain, and alcalase treatment for 4, 4, and 2 h, respectively, when these three hydrolysates possessed high fluorescence intensity (Fig. 3D–F). In other words, these hydrolysates with excellent antioxidant activity were rich in the exposed hydrophobic amino residues, which could access and trap the lipid-soluble DPPH radicals (Zhao et al., 2013; Zhu, Zhang, Kang, Zhou, & Xu, 2014). Above results show that DPPH radical scavenging activity of protein hydrolysates depended on the enzyme type and hydrolysis conditions employed, corresponding to the previous reports (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010; Klompong et al., 2007).

3.5.2. Metal ion-chelating activity

Given that certain transition metal ions can trigger the Haber-Weiss reaction and induce the generation of hydroxyl radical, we measured the Fe^{2+}-chelating ability of black bean hydrolysates. As described in Fig. 5D–F, Fe^{2+}-chelating ability of black bean hydrolysates exhibited effective chelating capacity for Fe^{2+} in a dose-independent manner, corresponding to the previous reports (Giménez et al., 2009; Naqash & Nazeer, 2013; Zhang et al., 2018). All the black bean protein hydrolysates under different reaction times presented Fe^{2+} chelating ability as the IC_{50} values ranging from 6.82 to 30.68 μg/mL, which were markedly lower than that of documented protein hydrolysates, such as hemp protein hydrolysate (IC_{50} = 1.6 mg/mL) (Tang, Wang, & Yang, 2009), rapeseed protein hydrolysate (IC_{50} = 3.93 mg/mL) (He, Girgih, Malomo, Ju, & Aluko, 2013), and soybean hydrolysate (IC_{50} = 0.67 mg/mL) (Zhang et al., 2018). The ficin hydrolysate showed the lowest IC_{50} value (10.26 μg/mL) at the hydrolysis time of 2 h (Fig. 5D), while the strongest chelating ability was found in black bean protein after 180 min bromelain treatment (Fig. 5E). Compared with black bean protein hydrolysates obtained from plant proteases, alcalase-treated hydrolysates under different reaction time exerted the strongest chelating capacity for Fe^{2+} (Fig. 5F). This might attribute to the specificity of enzymes employed. Alcalase is an alkaline

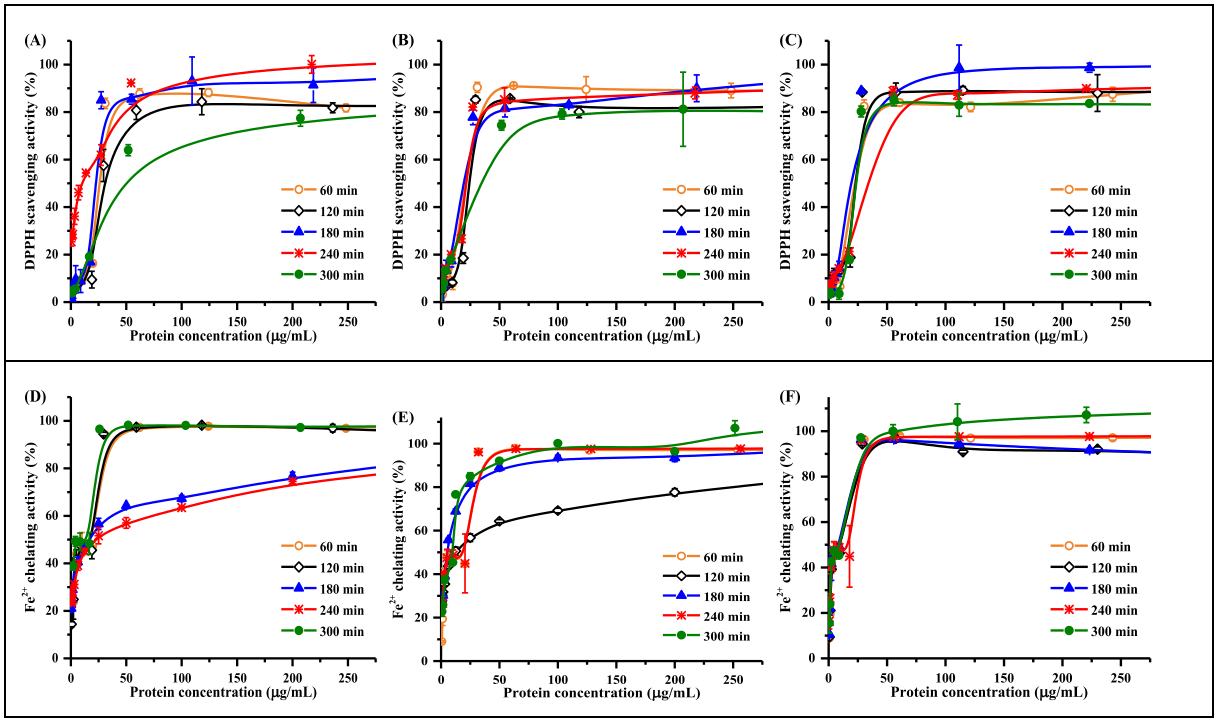

Fig. 5. *In vitro* antioxidant activities of black bean protein hydrolysates. DPPH radical scavenging activity of black bean protein hydrolysates prepared with ficin (A), bromelain (B) and alcalase (C); Metal ion-chelating activity of black bean protein and hydrolysates prepared with ficin (D), bromelain (E) and alcalase (F).

endopeptidase that has broad specificity and cleavage peptide bonds effectively (Thiansilakul, Benjakul, & Shahidi, 2007; van der Ven, Gruppen, de Bont, & Voragen, 2002). Peptide cleavages elevated the amounts of carboxylic and amino groups in branches of the acidic and alkaline amino acids, favoring Fe^{2+} binding and thus retarding the oxidation reaction (Dong et al., 2008).

3.5.3. Antioxidant activity of black bean hydrolysates on lipid oxidation

Given that the high hydrophobicity and antioxidant activity of hydrolysates might be favorable to oil stability, we selected three protein hydrolysates prepared from the treatment of ficin (FPH), bromelain (BPH) and alcalase (APH) for 240, 240 and 300 min, respectively. Ferric thiocyanate method, measuring the peroxide levels during the initial stage of linoleic acid oxidation, was firstly used to evaluate the antioxidative effects of protein hydrolysate on lipid oxidation. As depicted in Fig. 6A, OD values of control increased rapidly at the first two days, indicating the formation of primary products in linoleic acid autoxidation. The absorbance of control subsequently decreased possibly due to the peroxide decomposition (Caetano-Silva et al., 2018). At the presence of these three selected hydrolysates, OD values were kept below 0.12 through the experimental period. The strongest inhibition activity of linoleic acid peroxidation was 94.70 ± 0.27%, demonstrating the prominent capacity of protein hydrolysates on suppressing the formation of peroxides. Intriguingly, all these three hydrolysates presented similar inhibitory behaviors in linoleic acid system, while the lowest absorbance after 7 days was observed in BPH. Accordingly, linoleic acid peroxidation could be effectively suppressed by all these investigated hydrolysates, particularly the bromelain-treated hydrolysate.

Since these three selected black bean protein hydrolysates exhibited excellent abilities to inhibit linoleic acid oxidation, their antioxidant potential in sunflower oil were then investigated by using rancimat stability assay. As shown in Fig. 6B, all the three tested hydrolysates had the capacity to delay the induction period of sunflower oil.

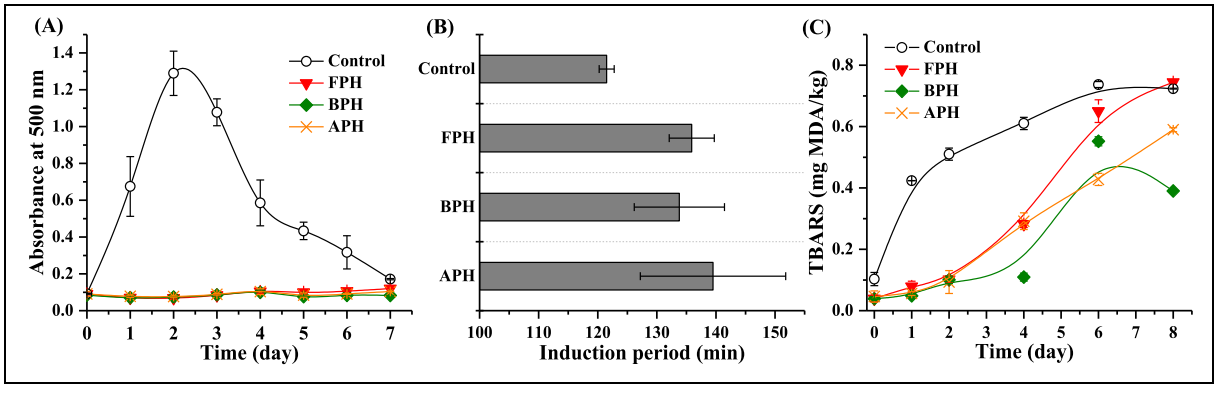

Fig. 6. Antioxidant activity of three black bean hydrolysates in lipid oxidation. (A) Inhibition activity of protein hydrolysates on linoleic acid peroxidation; (B) Induction time (min) for Rancimat stability test at 120 °C for sunflower oil with hydrolysates; (C) Thiobarbituric acid reactive substances (TBARS) of sunflower oil O/W emulsions containing hydrolysates. FPH: black bean protein treated with ficin for 240 min; BPH: black bean protein treated with bromelain for 240 min; APH: black bean protein treated with alcalase for 300 min; Control: sample without the addition of hydrolysates.

Specifically, FPH and APH increased the induction time of sunflower oil from 2.02 ± 0.02 h to 2.27 ± 0.06 h and 2.33 ± 0.21 h, respectively ($P < 0.05$). Similarly, BPH slowed down oxidation up to 133.80 ± 7.64 min, performing the potential of retarding the lipid oxidation.

Since these three hydrolysates exhibited the antioxidant potential in oil, we reasoned that black bean protein hydrolysates might play positive roles in oxidative stability of the O/W emulsion. To confirm this, effects of these three samples on the oxidative stability of the O/W emulsion were evaluated by TBARS test, as shown in Fig. 6C. All the emulsions on day 0 exhibited lower TBARS production, which then increased significantly as the storage progressed. Similar patterns were found in the previous reports about the oxidative stability of emulsion, such as O/W emulsion containing rice bran protein hydrolysates (Cheetangdee & Benjakul, 2015), potato protein hydrolysate (Cheng, Xiong, & Chen, 2010), and phenolic compounds (Maqsood & Benjakul, 2010). Intriguingly, the addition of these three tested hydrolysates could remarkedly decline the TBARS levels compared with the emulsion without hydrolysates. For instance, O/W emulsion with the addition of BPH showed lower TBARS levels at the first 4 days of storage period, after which TBARS formation was slightly enhanced. Both FPH and APH significantly suppressed rate of TBARS formation in a time-dependent manner. At the end of the storage period, i.e. 8d, BPH exhibited the highest efficacy in preventing the increase in TBARS level in comparison with others, corresponding to the results of inhibiting linoleic acid oxidation (Fig. 6A). This might ascribe to the higher radical scavenging activity and metal chelating ability of bromelain-treated hydrolysate, thereby reducing the subsequent generation of free radicals and further retarding the radical-mediated oxidative chain reactions (Maqsood et al., 2010).

The emulsion stability was further confirmed by the microstructural observation of the emulsions using optical microscopy (Fig. S1). All the emulsions kept stable during the first 4 days storage, while these emulsions containing protein hydrolysates were observed to undergo certain degrees of coalescence upon 8 days of storage. This may be interpreted as competitive adsorption of Tween 20 and protein hydrolysates at the droplet interfaces (Adjonu et al., 2014; Cheng et al., 2010). The small peptides in protein hydrolysates may also adsorb weakly to droplet interfaces, and therefore will be gradually desorbed during storage, allowing droplets to recoalesce and aggregate. Similarly, at the last storage time studied, emulsions containing FPH and APH showed more and larger droplets than that containing BPH, which might be correlated to the higher proportion of large peptides and hydrophobic residues in the latter (Joye & McClements, 2014; van der Ven, Gruppen, de Bont, & Voragen, 2001).

4. Conclusion

This study investigated the physicochemical properties and antioxidant activities of black bean protein hydrolysates produced by ficin, bromelain or alcalase under different hydrolysis time. Results showed that black bean protein hydrolysates obtained by treatment with bromelain or alcalase presented higher DH than that obtained by ficin. All the black bean hydrolysates under different hydrolysis times exhibited excellent DPPH radical scavenging activity, and the IC_{50} values of Fe^{2+} chelating ability ranged from 6.82 to 30.68 μg/mL. Three selected protein hydrolysates (FPH, BPH and APH) were evidenced for their excellent capacity on delaying the induction period of sunflower oil. Noticeably, these three hydrolysates, especially BPH, could significantly suppress the peroxides formation in linoleic acid system and enhance the oxidative stability of the oil-in-water emulsion, which might due to their high hydrophobicity and emulsifying properties. These results strongly support the potential use of black bean protein hydrolysates to prolong the shelf life of oil-rich food.

Declaration of interests

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2019.105222.

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