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The effect of enzymatic hydrolysis on the antioxidant activities and amino acid profiles of defatted chia (*Salvia hispanica L.*) flour

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Abstract

The aim of this study was to determine the effect of enzymatic hydrolysis using different proteases (Alcalase® and papain) and hydrolysis period on antioxidative activities and amino acid profiles of hydrolysed chia peptides. The experiment has been carried out using a completely randomized block design. The protein from defatted chia flour (DCF) was first isolated using different extraction pH (pH 10; 11; 12) and precipitation pH (pH 3.5, 4.0 and 4.5) to determine the highest protein isolated (CI) yield. The chia isolate (CI) extracted using the combination treatment (pH 12, 3.5) demonstrated the highest protein content of 17.22% and was selected to further hydrolysed using Alcalase® and papain enzyme at different hydrolysis time. The degree of hydrolysis (DH), protein solubility and peptide content of the chia protein hydrolysates (CH) were observed. Alcalase®-CH and Papain-CH demonstrated the highest DH at 60 mins of hydrolysis with the value of 47.09% and 44.29%, respectively. The protein solubility and peptide content were directly proportional to the DH. The Alcalase®-CH hydrolysed at 60 mins exhibited the highest antioxidant activities as measured by DPPH, ABTS and FRAP assays with values of 35.46μM AAE, 34.45μM TE and 23.11 μM $FeSO_4.7H_2O$ E, respectively. The Alcalase®-CH demonstrated higher ($p<0.05$) hydrophobic amino acid (42.51%) compared to and Papain-CH (37.25%,). The highest aromatic amino acid content also recorded by Alcalase®-CH (20.10%), whereas Papain-CH with the value of 15.54%. However, both CH exhibited higher hydrophilic and aromatic amino acid compared to DCF and CI. This result has proved that the enzymatic hydrolysis of CH using Alcalase® and papain improved the nutritional and antioxidant capabilities, thus potentially represent a naturally occurring antioxidant ingredient in the production of functional food and nutraceutical appliance with significant health benefits.

1. Introduction

Free radicals are freely formed during normal metabolic processes, and they are a major factor in the development of diseases such as cancer and other cardiovascular diseases (Lobo *et al*., 2010). The human body can synthesize its defence system against the free radicals but not sufficiently effective to prevent the damage (Makinen *et al*., 2012). Hence, the need for consumption of antioxidant-rich food such as antioxidative peptides basically to protect the human body against oxidative damage and consequently associated diseases. Many synthetic antioxidants are used as a free radical scavenger in the food and pharmaceutical industries (Najafian and Babji, 2012). However, they cannot proffer total protection against oxidative stress (Poljsak *et al*., 2013), and are viewed to have a possible toxic effect which has spurred research interest especially in the discovery of natural antioxidants from plant foods which are more compatible with human nature (Taghvaei and Jafari, 2015).

Some food-derived proteins have potential as a natural source of producing antioxidative peptides. However, these proteins are inactive in the intact form within the food matrix due to their large size and conformational arrangement that buries hydrophobic or reactive groups inside the protein structure (Garcia *et al*., 2013). Enzymatic hydrolysis is a process that was highly potential to release the bioactive peptides from parental protein and exhibiting various biological activities and nutritional (Lee *et al*., 2010). Bioactive peptides exhibit different physicochemical properties and biological activities, depending on their amino acid composition

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(Kim and Wijesekara, 2010). The antioxidative peptides contain 2 to 20 amino acid units and usually are inactive within the sequences of their parent protein but can be released by enzymatic hydrolysis (Shahidi and Zhong, 2008).

Chia seed (*Salvia hispanica L*) is a herbaceous plant belonging to the mint family (Munoz *et al*., 2013) and often referred under its common name 'chia'. It is one of the non-conventional protein sources which have been increasingly studied in recent years (Fernández-López *et al*., 2018). Generally, chia is rich in polyunsaturated fatty acids (PUFA) and lipophilic phytochemicals such as tocopherols or vitamin E (Nitrayova *et al*., 2014). Besides the oil component, in general, chia seeds comprise 17%–24% protein (Sandoval-Oliveros and Paredez-Lopez, 2013), make it a new potential of plant-based protein source. Chia seed protein contains four different fractions, among which globulins are the major components, and albumins, glutelins and prolamins are present in almost equal proportion (Sandoval-Oliveros and Paredez-Lopez, 2013). These proteins are readily digestible (Sandoval-Oliveros and Paredez-Lopez, 2013) and possess good foaming and excellent water and oil holding capacities (Olivos-Lugo *et al*., 2010). Chia seed proteins contain all the essential amino acids, and the percentage of glutamic acid, arginine and aspartic acid were very high (Sandoval-Oliveros and Paredez-Lopez, 2013). Currently, the utilization of chia focuses on the extraction of chia oil. Industrial production for chia oil generates waste, presently used in the manufacturing of defatted chia flour (DCF) and animal feed, these being low-cost products. This provides a new research area on the utilization of protein from the DCF industrial waste as a strategy used to add value to this by-product.

There are various good claimed on chia see, especially its benefits to human health. However, there are still not many published studies on the bioactivities of chia peptides produced via enzymatic hydrolysis, mainly the potential as an antioxidant. Thus, the objective of this study is to determine the antioxidant activities and amino acid profiles of chia hydrolysates enzymatically hydrolyzed using different protease enzymes (Alcalase® and papain) at a different time of hydrolysis. The enzymatic hydrolysis of CI using an Alcalase® and papain was expected to produce the CH with better nutritional quality and can be used as a new natural antioxidant plant-based protein source.

2. Materials and methods

2.1 Materials

The DCF was supplied by The Chia Co (Australia). The Alcalase® from *Bacillus lechinoformis* (≥2.4 U/g) and papain from *Carica papaya* (30 U/mg) were supplied by Sigma-Aldrich (USA). All other reagents used were of analytical grade were from Sigma Aldrich (USA).

2.2 Protein isolation

The method of protein isolation as reported by Lopez *et al*. (2017) was used to produce the CI. Before the protein extraction is performed, the DCF sample was first demucilaged. The DCF was mixed with distilled water (with a ratio of 1:20), stirred for 30 mins and centrifuged at 10,000 × *g* for 15 mins. The mucilaginous intermediate phase of the slurry was removed, while the chia proteins were recovered in the upper aqueous phase and the bottom phase. The demucilaged DCF was then dispersed in different pH of alkaline water (pH 10; pH 11; pH 12) using a DCF-to-water ratio of 1:20 to extract the protein. This slurry was stirred at ambient temperature for 1 hr using a magnetic stirrer to facilitate protein solubilization before centrifuged (Allegra 64R Centrifuge, Beckman Coulter Inc., USA) at 10,000 × *g* for 30 mins. The supernatant containing the dissolved protein was collected and further filtered using filter paper (Whatman No. 4) to remove any insoluble particles. The filtered supernatant was then acidified to pH 3, 4 and 4.5 to precipitate the dissolved protein. The protein precipitate was recovered by centrifugation and washed with Milli-Q water (3 × 50 mL) before re-suspended in Milli-Q water at pH 7.0. The mixture was stirred for 10 mins to solubilize the protein. This protein solution was lyophilized using a freeze dryer (Scanvac CoolSafe Touch 110-4, Labogene, France) and the protein powders obtained were collected. The freeze-dried chia protein isolated (CI) was packed in an airtight container and stored at 4°C until further analysis.

2.3 Protein quantification

Protein concentration was determined using Biorad dye reagent (Bio-Rad, Richmon, USA) according to Bradford (1976). A volume of 30 µL of each sample was added to 1 mL of the reagent, and the mixture was reposed for 5 mins before determined the absorbance using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan) at 595 nm. A bovine serum albumin (BSA) standard curve was prepared with a 2 mg/mL stock solution of bovine serum albumin (BSA).

2.4 Enzymatic hydrolysis

Chia protein hydrolysate (CH) were prepared via enzymatic hydrolysis by Alcalase® and papain enzyme at a different time of hydrolysis (15, 30, 60, 120 and 180 mins) according to the method reported by Chatterjee *et al. (*2015) with some modifications. The CI was soaked

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in distilled water, and the suspension was incubated with Alcalase® (1:100 w/w, pH 8, 60°C) or papain (1:100 w/w, pH 7, 60°C) before heated at 90°C for 5 mins for enzyme deactivation. All the CH samples were filtered with filter paper (Whatman No. 4), and the filtrates were lyophilized using a freeze dryer (Scanvac CoolSafe Touch 110-4, Labogene, France) for further analysis.

2.5 Degree of hydrolysis

The effect of hydrolysis time on the degree of hydrolysis (DH) was determined using the trichloroacetic acid (TCA) method with slight modification (Klompong *et al*., 2007). At the end of each hydrolysis time, an aliquot of 10mL of the aqueous suspension of enzyme-treated fractions was added to 10 mL of 20% trichloroacetic acid (TCA) to obtain 10% TCA-soluble nitrogen and 10% TCA-insoluble nitrogen and then centrifuged at 15,770 × *g* for 15 mins at 4°C. The supernatant was decanted and analyzed for soluble nitrogen content (N) using the Kjeldahl method (Kjeltec TM 2100, Foss, Denmark). The percentage of DH is expressed as follows.

$$\text{DH (\%)} = \frac{\text{Soluble N in TCA 10\% (w/v)}}{\text{Total N in the sample}} \times 100$$

2.6 Peptide content

A rapid, sensitive and convenient o-phthaldialdehyde -(OPA-) based spectroscopic assay with some modification was performed to measure proteolysis of CH in a buffered solution (Church *et al.,* 1983). A fresh OPA solution was prepared as follows: a volume of 25 mL sodium tetraborate solution (100 mmol/L) was mixed with 2.5 mL of 20% (w/v) sodium dodecyl sulfate solution, 40 mg of OPA solution (dissolved in 1mL of methanol) and 100 µL of ß-mercaptoethanol. The volume was adjusted to 50 mL with deionized water. The reagent was then placed in a vial covered with aluminium foil to protect it from light. The amount of 50 µL of each CH was mixed with 2 mL of OPA reagent and incubated for 2 mins at ambient temperature. The absorbance at 340 nm was measured with the spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). Casein tryptone in phosphate buffer pH 7.4) was used as a standard to quantify the peptide content.

2.7 Protein solubility

The soluble protein component of CH was determined according to the method of Bradford (1976) based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 (in the dye reagent) shift from 465 nm to 595 nm when binding to the protein occurs. The dye reagent was prepared by diluting 1 part of concentrated Bio-rad dye reagent (Bio-Rad, Richmon, USA) with 4 parts of distilled water and filtered through a Whatman No. 4 filter paper to remove particulates. An aliquot of 100 µL sample was added to 5 mL diluted dye reagent and allowed to stand for 5 mins at room temperature. The blue protein-dye form was being measured at 595nm using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). The soluble protein content was quantified using bovine serum albumin, BSA.

2.8 Antioxidant activities

2.8.1 DPPH radical scavenging assay

The DPPH scavenging activity of CH was measured using the method described by Ahn *et al*. (2014) with a slight modification. Briefly, a 70 µL of CH was mixed with 70 µL of DPPH solution (150 µM in MeOH) followed by standing at room temperature for 30 min in the dark. The absorbance was measured by a microplate reader (Tecan GENios, Austria GmbH, Austria) at 517 nm. The DPPH radical scavenging activity was expressed by the ascorbic acid equivalent (AAE) using an ascorbic acid standard curve (10–50 µM).

$$\text{DPPH radical scavenging activity} = \frac{\text{(Absorbance control} - \text{Absorbance sample)}}{\text{Absorbance control}}$$

2.8.2 ABTS radical scavenging assay

The ABTS radical stock solution was prepared according to the method described by Ahn *et al*. (2014), and the working solution (absorbance with 1.5 at 414 nm) was prepared by dilution). A 50 µL of sample and 150 µL of ABTS+ were mixed, stand for 10 mins, and the absorbance was determined by a microplate reader (Tecan GENios, Austria GmbH, Austria) at 414 nm. The scavenging activity was expressed by µM Trolox equivalent (TE) using a Trolox standard curve (10–50 µM).

$$\text{ABTS radical scavenging activity} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}$$

2.8.3 Ferric reducing antioxidant power (FRAP) assay

The procedure described by Guo *et al*. (2003) was followed to determine the FRAP activity of the CHbased on the principle reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form in the presence of antioxidants. Briefly, the FRAP reagent contained: 2.5 mL of 10 mmol/L TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3$ and 25 mL of 0.3 mol/L acetate buffer (pH 3.6) was prepared freshly. An aliquot of 40 µL of CH sample was mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. The absorbance of the reaction mixture was spectrophotometrically measured at 593nm after incubation at 37°C for 10 mins using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan).

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The $FeSO_4$ was used as the standard solution. The result was expressed as µM $FeSO_4.7H_2O$ equivalent.

2.9 Amino acid profile

The amino acid composition of CH was measured according to Ghassem *et al.* (2014). The CH samples were mixed with 6M hydrochloric acid at 110°C for 24 hrs to hydrolyze the protein. The internal standard a-aminobutyric acid was added to the hydrolyzed samples and filtered with a 0.2 mm cellulose acetate membrane filter (Whatman No. 1). The derivations of the amino acids were performed at 55°C for 10 mins in a heating block. The separations of the amino acids were performed using a C18 AccQ-Tag amino acid analysis column (3.9 x 150 mm, Waters, USA) with the temperature-controlled at 37°C and a flow rate set at 1 mL/min. The UV detector was operated at 248 nm (for peak identification), and the excitation and emission wavelength for the fluorescence detector was 250 and 395nm (for amino acid quantification), respectively. The number of amino acids was calculated, based on the peak area in comparison with that of the standard. Alkaline hydrolysis was done for determination of Tryptophan level.

2.10 Statistical analysis

The analyses were conducted in triplicate. The one-way and two-way analysis of variance (ANOVA) was performed, and the means were compared using Duncan's multiple range test. The data were expressed as the mean±standard deviation. The significance value ($p<0.05$) between samples was determined using Statistical Analysis Software (SAS) 9.4.

3. Results and discussion

3.1 The protein content of chia protein isolated (CI)

Before undergoing the process of enzymatic hydrolysis, the chia protein was first being isolated from the chia flour using the alkaline extraction and isoelectric point precipitation method. This method was an efficient method to allow protein separation (Pedroche *et al.*, 2004). Figure 1 summarizes the protein content of chia protein isolated (CI) at different alkaline extraction and isoelectric precipitation pH. The protein content of CI was varied between 7.35% to 17.22%. These relatively low yields could be related to the native content, and biochemical characteristics of the proteins present in chia flour. According to the previous report, the protein content of defatted chia flour is between 18% to 27% (da Silva *et al.,* 2017; Cardenas *et al*., 2018; Hernandez-Domingues *et al*., 2019). Based on the result, the highest protein content was obtained at the high extraction pH (pH 12, 3.5) with the value of 17.22%, while the lowest protein content was recorded by the lowest extraction pH (pH 10, 4.5) with the value of 7.35%. Alkali might aid protein extraction by increasing the protein solubility. Generally, protein in DCF was not soluble. The solubility of these proteins can be enhanced with the increase of pH by adding alkali. The alkalization of the medium by adding NaOH will cause rupture of the disulphide bond, thus improving the protein solubility (Damodaran, 1996). The previous report by Buning *et al*., (2009) also shown the similar trend where there was a significant increase in protein recovery of cottonseed protein isolates in the more alkaline extraction medium (pH 12.5) up to 70%. However, the result from Figure 1 showed that the pH effect on isoelectric precipitation did not exhibit a characteristic pattern within treatments, suggesting no significant correlation between the protein concentrations in relation to the isoelectric precipitation. This result contradicted the previous report by Vilg and Undeland (2017), and Liu *et al*. (2013) stated that the isoelectric precipitate pH gives the significant effect to the protein recovery of brown seaweed and wheat germ sample, respectively.

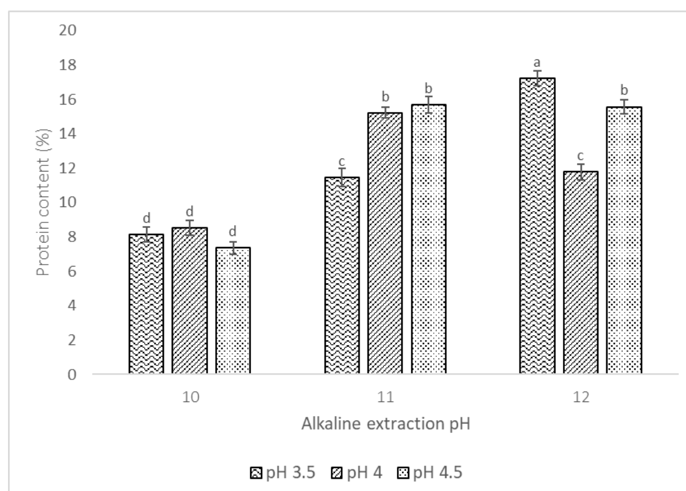

Figure 1 The protein quantification of chia protein isolates (CI) at different alkaline extraction pH and isoelectric precipitation pH. Different letters on the bars indicate statistical significant difference ($p<0.05$) of the protein content (%).

3.2 The degree of hydrolysis (DH), protein solubility and peptide content of the chia protein hydrolysates (CH)

The highest protein content of CI was selected for further enzymatic hydrolysis. The enzymatic hydrolysis of CI was carried out to obtain the degree of hydrolysis (DH) at which was biologically active. Table 1 demonstrated the effect of hydrolysis time on the DH, protein solubility and peptide content of chia protein hydrolysates (CH) using two different proteases: Alcalase® and papain. The DH was influenced by the hydrolysis reaction period and the ability of the proteases to carry out the proteolysis.

As shown in Table 1, a rapid increase in DH occurs

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Table 1. The degree of hydrolysis, protein solubility and peptide content of chia protein hydrolysates at different time of hydrolysis

Samples	Time of Hydrolysis (min)	Degree of Hydrolysis (%)	Protein solubility (µg/g)	Peptide content (µg/g)
Defatted Chia Flour (DCF)	-	-	2.46±0.19^{I}	7.12±0.41^{H}
Chia Protein Isolated (CI)	-	-	19.03±0.22^{H}	27.62±0.64^{G}
Chia hydrolysates hydrolyzed using Alcalase® (Alcalase®-CH)	15	16.28±0.45^{G}	45.39±0.84^{F}	59.71±1.02^{E}
	30	32.68±0.32^{D}	68.01±0.78^{C}	90.19±0.76^{C}
	60	47.09±0.86^{A}	83.71±1.06^{A}	104.46±0.58^{A}
	120	46.83±1.22^{A}	83.27±0.88^{A}	104.11±0.36^{A}
	180	45.60±1.10^{B}	79.91±0.47^{B}	103.65±1.29^{A}
Chia hydrolysates hydrolyzed using papain (Papain-CH)	15	10.22±0.71^{H}	39.21±0.41^{G}	51.43±0.89^{F}
	30	22.52±0.69^{F}	59.90±0.99^{E}	69.98±0.76^{D}
	60	44.29±1.01^{B}	79.98±0.55^{B}	95.72±0.50^{B}
	120	31.79±0.42^{E}	79.25±1.02^{B}	89.70±0.69^{C}
	180	39.02±0.91C	65.22±0.60^{D}	89.92±0.56^{C}

Values shown are the mean±standard deviation of three replicates. Values in the same column without a common superscript letter are significantly different (p<0.05)

during the initial phase of hydrolysis. However, the proteolysis rate of Alcalase®-CH tends to enter the plateau phase after 60 mins of hydrolysis and become decreases after 120 mins of hydrolysis. It was believed that at the initial stage of hydrolysis, the concentration of substrate and the enzyme activity were relatively higher, leading to a higher rate of bond cleavage and proteolysis. However, as the hydrolysis process continued until reached the reaction equilibrium, the rate of reaction become decreased (Zhang *et al*., 2012). This result indicates that the DH level is not solely affected by the length of hydrolysis. Several hypotheses from the previous study have explained this trend included a decrease in the concentration of peptide bond (Guerard *et al*., 2001), thermal inactivation of the enzyme (Demirhan *et al*., 2011a) and enzyme inhibition by hydrolysis products (Demirhan *et al*., 2011b). According to Demirhan *et al.* (2011b), the enzyme catalyst can occur in the reverse reaction. As the hydrolysis time increases, there is an accumulation of hydrolysis product, resulting in the reverse reactions and leads the enzymes to produce the substrate again, and consequently decrease the DH level. The highest ($p<0.05$) DH was recorded by the Alcalase®-CH (47.09%, 60 mins). For Papain-CH, the highest DH was recorded at 60 mins of hydrolysis with the value of 44.29%, a 2.8% lower ($p<0.05$) compared to the Alcalase®-CH. This result shows that the proteolysis reactions of breaking the CI to smaller peptides chains performed faster and more efficient with Alcalase® as catalyst compared to papain. The similar result was found previously by Li *et al*. (2015) on *Camilla oleifera* sample, where the DH of the sample that was hydrolyzed using Alcalase® showed significantly higher DH compared to papain with the value of 31.25% and 22.13%, respectively. Alcalase® is an endopeptidase enzyme, capable of hydrolyzing protein with a broad spectrum of peptides bond, especially those with large charge-free residues (Hrckova *et al.,* 2002). Besides the DH, the specific and unique enzyme active site is the other most significant factor affecting the resulting bioactivities mainly antioxidant. Therefore, all CH samples from both Alcalase® and papain treatment were further analyzed for antioxidant analysis.

Protein solubility is a key property of food proteins, and it is dependent on the distribution of hydrophobic and hydrophilic amino acids on the protein surface, and the thermodynamic interactions between proteins and water. The determination protein solubility using Bradford assay (Bradford, 1976) involved the binding of Coomassie Brilliant Blue G-25 dye to CH that causes a shift in the absorption maximum of the dye from 465 nm (protonated red cationic form) to 595 nm (stable unprotonated blue) (Reisner *et al.,* 1975). From Table 1, the value of protein solubility was directly proportional to the DH. According to Linares *et al.* (2000), the enzymatic hydrolysis process produced soluble peptides with both hydrophilic and hydrophobic properties, where the proportion of both are dependent on the DH levels. The enzyme reactions cause the secondary and tertiary structure of the protein to be revealed, which in turn favours the reaction between hydrophobic groups and increases the protein solubility (Nurfatin *et al*., 2014). The highest ($p<0.05$) protein solubility and peptide content were demonstrated by the Alcalase®-CH at 60 mins of hydrolysis with the values of 83.71 µg/g and 104.46 µg/g, respectively. This result indicated that, as the DH increased, the protein solubility and peptide content of CH also increased. The similar result was obtained by Morais *et al.* (2013) and Segura-Campos *et al*. (2012) with the sample of whey and *Vigna unguiculate* protein hydrolysates, respectively have

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reported that the increase of the protein solubility and peptide contents were directly proportional with the increase of DH.

3.3 The antioxidant activities of chia protein hydrolysates (CH)

3.3.1 DPPH radical scavenging activity

The DPPH radical scavenging assay has been widely used to evaluate radical scavenging activity of food extract because it is more stable than the conventional natural radicals (Chandrasekara and Shahidi, 2011). The assay is based on the reduction of methanolic DPPH radical solution at 517 nm in the presence of the hydrogen donating antioxidant, due to the formation of the non-radical DPPH-H by the reaction (Dong *et al*., 2015). The hydrogen-donating substance would scavenge the DPPH radical, and subsequently, the absorbance is reduced (Shimada, 1992). Based on this principle, the scavenging ability of CH hydrolyzed using Alcalase® and papain at different times of hydrolysis on the DPPH radicals were measured. The results are reported as the ascorbic acid equivalent (AAE) and are shown in Table 2. The DCF demonstrated the lowest ($p<0.05$) DPPH radical scavenging with the value of 4.45 µM AAE followed by the CI with the value of 9.19 µM AAE. This result showed that the protein from chia was naturally contained large molecules with limited accessibility of functional chains (R-groups) with reactive species and electron-dense peptide bonds. Nevertheless, the free radical scavenging capacity was significantly enhanced after enzymatic hydrolysis. The DPPH free radical scavenging activities of both Alcalase® and papain treatment were increased by the increasing of hydrolysis period up to 60 mins, with the highest values of 35.46 µM AAE and 33.09 µM AAE, respectively. This result indicated that the Alcalase® and papain catalyst was successfully released antioxidant peptides that are inactive when they are encrypted in the intact protein. According to Shahi *et al*. (2020), the antioxidant activity of the samples potentially enhanced by increasing the hydrolysis time following increasing the degree of hydrolysis and releasing more hydrophobic and active peptides and amino acids. The enzymatic hydrolysis releases more hydrophobic and shorter active peptides that highly responses to DPPH radicals, thus these compound have a higher capacity to be promptly accessible and inhibit the free radicals and assigned as an excellent antioxidant (Je *et al*., 2005; Kamdem and Tsopmo, 2019).

However, the extension of the hydrolysis time of both enzymes used represented a decrease in scavenging activity. The decreased of the scavenging activities after 60 mins of hydrolysis can be explained by the prolongation of hydrolysis process caused completed hydrolysis of peptides, leading to the released and high availability of free hydrophilic amino acids that are no longer active as an antioxidant (You *et al*., 2009). Besides that, the polarization also potentially reduced the scavenging capability of amino acids to DPPH radical (Zhao *et al*., 2010). The previous study by Li *et al*. (2015) and Shahi *et al*. (2020) on a sample of *Camilla oleifera* and *Bunium persicum Bioss* press cake, respectively showed that the proteolysis by Alcalase®, papain and pancreatin resulted in shorter peptides and generated potential antioxidative hydrolysates up to a

Table 2. The antioxidant activities of chia protein hydrolysates hydrolyzed using different proteases at the different time of hydrolysis

Samples	Time of incubation (hr)	DPPH Radical Scavenging Activity (µM ascorbic acid equivalent)	ABTS Radical Scavenging Activity (µM Trolox equivalent)	FRAP (µM $FeSO_4.7H_2O$ equivalent)
Defatted Chia Flour (DCF)	-	$4.45±0.24^J$	$3.79±0.16^I$	$0.26±0.22^J$
Chia Protein Isolated (CI)	-	$9.19±0.27^I$	$8.45±0.16^H$	$6.75±0.28^I$
Chia hydrolysates hydrolyzed using Alcalase® (Alcalase®-CH)	15	$19.96±0.32^G$	$20.08±0.27^F$	$8.53±0.41^H$
	30	$27.21±0.39^E$	$25.01±0.27^E$	$14.07±0.31^F$
	60	$35.46±0.32^A$	$34.45±0.31^A$	$23.11±0.31^A$
	120	$33.42±0.27^B$	$33.76±0.16^B$	$22.89±0.17^A$
	180	$33.18±0.46^B$	$29.35±0.21^C$	$21.26±0.18^B$
Chia hydrolysates hydrolyzed using papain (Papain-CH)	15	$15.42±0.52^H$	$18.51±0.39^G$	$8.49±0.15^H$
	30	$22.90±0.1^F$	$20.03±0.27^F$	$10.51±0.38^G$
	60	$33.09±0.41^B$	$29.39±0.28^C$	$20.16±0.25^C$
	120	$32.01±0.59^C$	$29.02±0.17^D$	$17.99±0.21^D$
	180	$30.98±0.61^D$	$29.05±0.27^D$	$16.69±0.25^E$

Values shown are the mean±standard deviation of three replicates. Values in the same column without a common superscript letter are significantly different ($p<0.05$)

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certain period and yet decreased with the prolongation of the hydrolysis process. CH hydrolyzed using Alcalase® showed significantly higher activity compared to papain. Alcalase® is an alkaline protease used to produce protein hydrolysates with better functional and nutritional characteristic than the original protein. According to Torucco-Uco *et al*. (2009), Alcalase® exhibited the subtilisin serine group in its active site, which has endopeptidase activity. Alcalase® hydrolyzed peptides with broad specificity, releasing peptides with antioxidant potential hydrophobic amino acids. Li *et al*. (2015) also reported that Alcalase® exhibited higher DPPH radical scavenging ability on the *Camellia sinesis L*. seed cake compared to papain with the EC50 values of 1.65 and 2.74, respectively.

3.3.2 ABTS radical scavenging activity

The antioxidative potential, cationic and water-soluble radical inhibitory ability of ABTS is dependent on the degree of hydrolysis and the amino acid composition of the peptide chains. Whereas, the sequences of the peptides are specifically dependent on the mechanism of actions of each protease enzyme (Sarmadi and Ismail 2010; Kamdem and Tsopmo, 2019). Table 2 exhibits the effect of different type of enzymes and time of hydrolysis on the ABTS scavenging activities reported as Trolox equivalent (TE). All the CH analyzed exhibited a higher radical scavenging ability compared to the DCF and CI. According to Table 2, the DCF and CI showed the lower ABTS radical scavenging potential, both with values of 3.79 µM TE and 8.45 µM TE, respectively. However, there were significant increases in ABTS radical scavenging capabilities with the increasing of hydrolysis time up to 60 mins for both Alcalase® and papain enzyme with values of 34.45 µM TE and 29.39 µM TE, respectively. During the enzymatic treatment, the release of electron and hydrogen donating properties of the active peptides from the intact protein might have led to an increase in ABTS scavenging activity (Pihlanto *et al*., 2008). These peptides could potentially react with the free radicals and convert them to more stable products and in turn stop the radical chain reaction (Pazinatto *et al*., 2013). However, after 60 mins of hydrolysis period, the radical scavenging activities tend to decrease ($p<0.05$). This happens because at this point most of the proteins or peptides are further degraded into inactive free amino acids, whereby these free amino acids are no longer function as an antioxidant (Samaranayaka and Li-Chan, 2011). According to the result reported by Tironi *et al*. (2010), the antioxidant capacity of peptides can decrease or completely lost after being change into free amino acids.

Among them, Alcalase® hydrolysate was the most potent for ABTS radical scavenging because it showed higher ($p<0.05$) activities during all the hydrolysis period. The ability of Alcalase® as a good radical scavenger is due to the nature of the Alcalase® itself as an endopeptidase which has a broad range of specificity towards the peptide bond especially residues without big charges (Rusnakova *et al.* 2002). The higher ABTS scavenging activity of the Alcalase® hydrolysates in comparison to other enzymes is confirmed in the previous literature. Alcalase® released peptides from flaxseed protein capable of scavenging ABTS more effectively than those obtained with papain and trypsin (Karamac *et al*., 2016). Ye *et al*. (2018) also found that the protein hydrolysates of carrot seed hydrolysate demonstrated the highest ABTS radical scavenging activities compared to those produced with papain, trypsin and neutrase.

3.3.3 Ferric reducing antioxidant power (FRAP)

The FRAP assay is a method that directly measures antioxidant in a sample and usually applied to evaluate the natural antioxidant ability to donate electron (Dorman *et al.,* 2003). It is deemed as a suitable assessment for total antioxidants in plants because the only compound that FRAP does not react with are thiols (Pyne *et al*., 2013). The method is based on the reduction of colourless ferric tripyridyltriazine (Fe^{3+}-TPTZ complex) to blue coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ complex) formed by the action of breaking radical change through donating a hydrogen atom (Rajurkar and Hande, 2011). The FRAP reducing power activity of CH hydrolyzed by Alcalase® and papain was demonstrated in Table 2. Among the samples, the DCF showed the lowest reducing ability with a value of 0.26 µM $FeSO_4.7H_2O$ equivalent. The unhydrolyzed CI also demonstrated a low reducing power with a value of 6.75 µM $FeSO_4.7H_2O$ equivalent. According to Xiao *et al*. (2014), the enzymatic hydrolysis caused an increase in free amino acids, and peptide fragments provide an additional source of electron and protons to maintain a high redox potential. After enzymatic hydrolysis by both Alcalase® and papain, the reducing power abilities significantly increased. The highest reducing power showed by Alcalase®-CH and Papain-CH were shown at 60 mins of hydrolysis with values of 23.11 and 20.16 µM $FeSO_4.7H_2O$ equivalent, respectively. In the other words, the FRAP reducing power of the CH obtained using Alcalase® and papain were about 3-folds higher than those of the non-hydrolyzed CI. Prolongation of hydrolysis resulted in the decreased of reducing power ability. According to Sbroggio *et al*., 2016), extensive hydrolysis would lead to the formation of shorter peptides (tri- and dipeptides) and free amino acids.

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Comparing these two enzymes, Alcalase® seen to have a higher capacity to release a high amount of hydrogen or electron-donating amino acid responsible for Fe^{3+} reduction compared to papain. The high ability of Alcalase®-CH is associated with the exposure of electron-dense amino acid side chain group (Thamnarathip *et al*., 2016), attributed to the specific peptide or amino acid composition produced by the enzyme (Wu *et al.* 2003). The values of FRAP reducing power of CH were comparable than those obtained for horse gram hydrolysates hydrolyzed with Alcalase® (Bashkar *et al*., 2019) and rice bran protein digested with the pepsin-trypsin system (Phongthai *et al.*, 2018).

3.4 Amino acid profile

The CH samples of each enzyme with the highest antioxidant activities were selected for amino acid profiling. The antioxidant properties of peptides are greatly influenced by the amino acid composition of the peptide (Malomo *et al*., 2014). The amino acid composition of the DCF, CI, Alcalase®-CH and Papain-CH were summarised in Table 3. The major amino acids found in CH were Glu, Arg, Asp, Ser, Phe, Gly and Thr. The essential amino acids in the Alcalase®-CH and Papain-CH were also found in relatively high amounts ($p<0.05$) with the total percentage of 41.07% and 38.76%, respectively compared to the DCF (33.71%) and CI (35.48%). The hydrophobic amino acids in the CH was significantly higher ($p<0.05$) compared to the DCF and CI with the highest was demonstrated by Alcalase®-CH with values of 42.51% followed by Papain-CH with the value of 37.25%. The increases of 8.69% and 3.43% respectively were due to the increasing values of Ala, Pro, Tyr, Val, Ile and Leu. The highest content of hydrophobic amino acid in CH has been expected because enzymatic hydrolysis generally exposes more protein chains, which increase the hydrophobicity. The antioxidant peptides could smoothly enter target organs through hydrophobic interactions with membrane lipid bilayers by the aid of their hydrophobicity, where they are able to exert significant capacity of scavenging radicals (Pouzo *et al*., 2016). The hydrophobic amino acids facilitated interactions with hydrophobic targets, such as the cell membrane, and thereby, enhance the bioavailability (Himaya *et al*., 2012). The hydrophobic amino acid such as Val and Ile responsible for forming a

Table 3. The amino acid profile of the defatted chia flour (DCF), chia isolated (CI) and chia hydrolysates (CH)

Amino acids	DCF (%)	CI (%)	Alcalase®-CH (%)	Papain-CH (%)
Asp	9.72±0.52^{A}	9.12±0.03^{B}	6.35±0.03^{C}	9.40±0.35^{BA}
Ser	7.40±0.22^{A}	6.86±0.12^{B}	6.35±0.29^{C}	4.64±0.06^{D}
Glu	18.46±0.31^{A}	18.07±0.14^{B}	12.24±0.95^{D}	17.13±0.21^{C}
Gly	5.57±0.12^{A}	5.09±0.17^{B}	5.12±0.08^{B}	4.70±0.14^{C}
His	3.52±0.09^{C}	3.80±0.04^{B}	4.93±0.58^{A}	3.20±0.10^{D}
Arg	12.69±0.05^{A}	12.00±0.15^{B}	11.20±1.02^{B}	9.71±0.26^{C}
Thr	5.43±0.01^{A}	4.50±0.02^{C}	4.68±0.01^{B}	3.55±0.37^{D}
Ala	3.33±0.11^{C}	3.24±0.02^{C}	4.08±0.29^{B}	4.52±0.29^{A}
Pro	3.72±0.14^{D}	4.09±0.02^{C}	4.64±0.07^{A}	4.29±0.05^{B}
Tyr	3.46±0.24^{C}	3.70±0.09^{C}	5.20±0.46^{A}	3.92±0.19^{B}
Val	4.00±0.11^{C}	4.60±0.08^{B}	4.97±0.04^{A}	4.69±0.11^{B}
Lys	4.64±0.08^{A}	4.39±0.15^{B}	2.87±0.05^{C}	4.42±0.10^{B}
Ile	1.69±0.13^{C}	1.97±0.01^{B}	4.17±0.40^{A}	4.02±0.22^{A}
Leu	4.01±0.08^{D}	4.29±0.02^{C}	6.92±0.13^{B}	7.22±0.29^{A}
Phe	5.94±0.11^{C}	6.63±0.01^{B}	7.20±0.08^{A}	4.14±0.35^{D}
Cys	0.88±0.05^{D}	0.94±0.03^{C}	0.98±0.03^{B}	2.93±0.02^{A}
Met	4.48±0.01^{B}	5.29±0.03^{A}	5.32±0.01^{A}	4.45±0.11^{B}
Trp	1.05±0.17^{C}	1.40±0.04^{B}	2.78±0.31^{A}	3.07±0.15^{A}
Total amino acid (%)	100	100	100	100
Total EAA[1] (%)	33.71±0.39^{D}	35.48±0.22^{C}	41.07±1.11^{A}	38.76±0.09^{B}
Total AAH[2] (%)	30.63±0.21^{D}	33.82±0.04^{C}	42.51±1.32^{A}	37.25±0.27^{B}
Total AAR[3] (%)	13.97±0.38^{C}	15.54±0.16^{B}	20.10±1.26^{A}	14.33±0.51^{C}

Values shown are the mean±standard deviation of three replicates. Values in the same column without a common superscript letter are significantly different ($p<0.05$).

[1]EAA = Essential amino acid (His, Thr, Val, Lys, Ile, Leu, Phe, Met, Trp)

[2]HAA = Hydrophobic amino acid (Ala, Val, Met, Ile, Leu, Phe, Pro and Tyr)

[3]AAR = Aromatic amino acid (Phe, His, Trp and Tyr)

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favourable hydrophobic micro-environment for peptide molecule (Bougatef *et al*., 2010). The highest hydrophobic amino acid content of the Alcalase®-CH has supported the result of antioxidant that has been discussed previously. The hydrolysates containing the higher amount of hydrophobic amino acids such as Leu, Ala, Pro and Phe, as well as other amino acid groups such as Tyr, Met and His, has been shown to enhance the potency of antioxidant peptides through proton-donation ability, electron-donation ability, and direct lipid radical scavengers (Sarmadi and Ismail, 2010; Samaranayaka and Li-Chan, 2011). Previous studies have been reported that the high content of hydrophobic amino acids found in the *Moringa oleifera* protein hydrolysates by Alcalase® with a value of 39.64% was one of the factors contributing to high antioxidant capabilities of the peptides (Aderinola *et al*., 2018).

The total percentage of aromatic amino acid in the Alcalase®-CH samples also increased significantly ($p<0.05$) after the enzymatic hydrolysis with the increasing value of 4.56%. Aromatic amino acids with large side group such as His and Trp contribute to the antioxidant potency of peptides because they act as hydrogen donors (Nam *et al*., 2008). According to Sarmadi and Ismail (2010), the aromatic amino acids (Phe, His, Tyr and Trp) are generally known as a positive free radical scavenger which can convert the free radicals to the stable molecules by donating electrons while retaining their stability through resonance structures increasing the radical-scavenging properties of the amino acid residues. This finding also has been supported by Rong *et al*. (2012) reported that the content of aromatic amino acids (Trp, Phe and Tyr) in the rapeseed protein hydrolysates fraction significantly increased ($p<0.05$) to 15.88% compared to unhydrolyzed sample (13.33%).

4. Conclusion

The results demonstrated that the highest CI was being isolated at the combination of extraction pH 12 and the precipitate pH 3.5. Two different protease enzymes (Alcalase® and papain) were used for the hydrolysis of CI. The enzymatic hydrolysis using Alcalase® and papain have significantly ($p<0.05$) increased the DH, protein solubility, peptides content, and subsequently succeeded in enhancing the antioxidant capability of CH. The highest antioxidant activity was demonstrated by Alcalase®-CH at 60 mins of hydrolysis as determined via DPPH and ABTS radical scavenging assay and FRAP reducing power assay. The higher antioxidant activities were due to the increased of hydrophobic and aromatic amino acids in the CH. This result proved that the enzymatic hydrolysis of CI using Alcalase® and papain improved the nutritional and antioxidant capabilities compared to unhydrolyzed CI, thus represent a naturally occurring antioxidant ingredient in the production of functional food and nutraceutical appliance with significant health benefits.

Conflict of interest

The authors declare no conflict of interest.

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