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RESECH PAPER

Evaluation of the *in vitro* α-Amylase Enzyme Inhibition Potential of Commercial Dried Laver (*Porphyra* Species) Seaweed Protein Hydrolysate

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Abstract

Modulating α-amylase enzyme activity with bioactive peptides of protein hydrolysate is one of a viable strategy in the management of diabetes through the control of postprandial glucose level. In this study, four proteolytic enzymes (pepsin, neutrase, alcalase and trypsin) were used to investigate the *in vitro* α-amylase inhibition of dried laver seaweed (*Porphyra* species) protein hydrolysates. Pepsin hydrolysate showed effective inhibition rate at an IC_{50} value of 1.86 mg/mL protein as compared to other enzyme hydrolysate. This hydrolysate was fractionated in to three groups in molecular weight (MW) cut-off of 5 and 10 kDa (MW<5kDa, MW = 5-10kDa and MW>10kDa). The MW<5kDa showed better inhibition rate with IC_{50} value of 1.18mg/mL. This fraction (MW<5kDa) was further separated to three fractions (F-I, F-II & F-III) using gel chromatography on Sephadex G-25. F-III achieved higher α-amylase inhibition at an IC_{50} value of 0.87mg/mL. The MW distribution of F-III showed the smallest MW fractions of 90-1000Da. It can be concluded that pepsin hydrolysate of seaweed protein demonstrated a high potential in inhibiting a α-amylase activity, and thus, could be used as an ingredient for development of functional foods having anti-hyperglycaemic effect.

Keywords: Seaweed, dried laver, protein hydrolysate, bioactive peptides, α-amylase inhibition.

Introduction

The burden of diabetes mellitus is enormous, due to its increasing global prevalence and the occurrence of chronic complications affecting a number of tissues including retinopathy, nephropathy, neuropathy, and cardiovascular disease, which results in high direct and indirect costs (Lapolla, Molin, & Traldi, 2013). Diabetes occurs in 366 million people worldwide, and this figure is expected to increase to 552 million people by the year 2030. Out of this, an estimated number of 25.8 million people have this disease only in the United States (Anguizola *et al*., 2013). Diabetes is a metabolic disorder characterized by high plasma glucose levels and classified as Type I and II. Type-II diabetes is the most prevalent form of diabetes , accounting for 90-95% of cases (Chiara & Adrianna, 2016). Available therapies for treating type-II diabetes consist of stimulation of endogenous insulin secretion, increase the activity of insulin at target tissues as well as inhibition of α-amylase enzyme to reduce the degradation of starch to decrease glucose production (Dastjerdi, Namjoyan, & Azemi, 2015; Kim, Rioux, & Turgeon, 2014; Kim, Nam, Kurihara, & Kim, 2008).

There are some antidiabetic drugs, such as acarbose, miglitol and voglibose, which inhibit α-amylase activity (α-AI) in recent days. However, these drugs are associated with undesirable side effects possessing negative outcomes on human health (Lordan, Smythb, Soler-Vila , Stanton, & Ross, 2013). Therefore, there is a need for natural α-amylase inhibitors that have minimal unwanted secondary effects. In view of addressing this , many research efforts have been made to search for more effective and safe α-amylase inhibitors from natural sources to develop functional food with various physiological activities such as antidiabetic effects (Wanga, Dub, & Song, 2010). Among numerous potential natural sources, seaweeds produce a variety of biologically active components with different structures and interesting functional properties.

The α-amylase and α-glucosidase inhibition properties of solvent extracts of several red, brown and green macro algae have been reported by numerous authors from elsewhere (Chu & Phang, 2016; Heo, Park, Lee, & Jeon, 2005; Kim *et al*., 2014; Lordan *et al*., 2013; Pai-An, Shih-Yung, Yu-Lan, Yi-Kuan, & Zwe-Ling, 2013; Schultz Moreira *et al*., 2014; Xindi *et al*., 2016; Yuan & Walsh, 2006). In

addition, the antihypertensive, Angiotensin-converting enzyme (ACE) inhibiting properties and antioxidant activities of seaweed protein hydrolysates and peptides has been reported in the previous studies (Beaulieu, Sirois, & Tamigneaux, 2016; Ko *et al*., 2012; Qu *et al*., 2015; Qu *et al*., 2010; Sheih, Fang, & Wu, 2009). Proteins that inhibit α-amylases have also been isolated from plants and microorganisms (Svensson, Fukuda, Nielsen, & Bonsager, 2004). Yua, Yin, Zhao, Liu and Chen, (2012) evaluated and validated the anti-diabetic activity of bioactive peptides from albumin against α-glucosidase and α-amylase. Fitzgerald *et al*. (2012) has also isolated and characterized bioactive Pro-Peptides with *in vitro* Renin inhibitory activities from the Macro algae *Palmaria palmata*. Thus far, there appears to be limited information regarding to α-amylase inhibition activity by seaweed protein derived-hydrolysates /peptides.

Macro algae, in particular, the red species, are reported to contain significant levels of protein (Fleurence, 2004). Laver *(Porphyra* spp.*)*, red algae, is one of the most widely consumed edible seaweeds (Hwang, Ki, & Chung, 2013). Despite the fact that most commercial dried laver (*Porphyra* species) contains a high amount of protein, up to 47% (Cian, Drago, de Medina, & Martínez-Augustin, 2015; Fleurence, 2004), few studies have been conducted on the α-amylase inhibitory potential of its protein hydrolysate. Exploring dried laver protein hydrolysates/peptides with functional α-amylase inhibitory activities provides an opportunity for the development of anti-diabetic functional food ingredients.

Therefore, the objectives of the present study were to: i) screen effective enzyme (s) for hydrolysis in producing α-amylase inhibitory hydrolysate from commercial dried laver protein ii) evaluate the α-amylase inhibitory potential of the protein hydrolysate produced by using the selected enzyme, iii) study the molecular weight distribution of the potent α-amylase inhibitory peptides produced under optimum conditions of the selected enzyme.

Materials and Methods

Materials

Commercially dried laver (*Porphyra* species) was obtained from Rudong Laver farming and processing Industry (Nantong, Jiangsu, China). The dried laver was pulverized by using laboratory mill (IKA model A11BS25, IKA Laboratory Technology, Staufen, Germany) to the particles size of less than 150 μm. The powder samples were kept in a sealed plastic bag and stored at -20°C until used for biochemical analysis. The commercial enzymes (Alcalase, Trypsin, Neutrase, and Pepsin) and the gel, Sephadex G-25 were purchased from Sigma-Aldrich Chemical Co. (Shanghai, Jiangsu, China). The other chemicals and reagents used were analytical grade.

Protein Extraction

Crude protein was extracted and prepared using the method described (Fleurence, Le Coeur, Mabeau, Maurice, & Landrein, 1995) with slight modification. Briefly, powdered commercial dried laver (particle size<150 μm) was added into deionized water (1:20, powder: water ratio) to allow cell lysis. The suspension was gently stirred overnight using a magnetic bar at 4°C. After incubation, the suspension was centrifuged (Avanti J-25 Centrifuge, Beckman Coulter, USA) at 10,000×g, 4°C for 45 min and the supernatant was collected. The pellets were re-suspended in sodium hydroxide (NaOH = 0.1M) in the presence of mercaptoethanol (0.5% v/v). The mixture was gently stirred at room temperature for 1hr before centrifugation (10,000xg, 45 min) and the extraction procedure was repeated on the pellet. Both supernatants were pooled. Extractions were performed in triplicate (N=3). The protein present in the supernatants was collected by isoelectric precipitation at pH 3.5-4.0. Following pH adjustment (pH 3.5 and 4.0), samples were allowed to stand for 60 min in the ice bath (0-4°C). The pellet obtained after centrifugation at 5000×g for 30 min 4°C was washed with acidic H_2O (pH 3.5-4) two times and centrifuged, then pellet was dissolved in distilled water, adjusted to pH 7, freeze-dried, powdered and stored at −20°C until further use as a substrate for enzymatic hydrolysis.

Determination of Protein and Amino Acid Profile

Total protein content was determined by taking 0.5g powder of dried laver using the micro-Kjeldahl method of total nitrogen determination. The total protein content was calculated by multiplying the total nitrogen content of the laver with a conversion factor of 6.25. The result was expressed in percentage (%) of sample dry weight.

The amino acid profile of the laver protein was determined by the method reported (Chamba, Hua, Murekatete, & Chen, 2015, Pastor-Cavada *et al*., 2013) using reverse-phase HPLC (RP-HPLC) (HP-Agilent 1100 model, Agilent Technologies) in the following settings: Samples (100 mg) were hydrolyzed with 8 mL of 6 $mol.L^{-1}$ HCl. The solutions were sealed in tubes under nitrogen for 3 min and incubated in an oven at 120°C for 22 h. The sample was injecting in to Agilent Octyldecylsilane (ODS) Analytical HPLC column (4.6 x 250mm, 5 μm particle size) (Agilent Technologies, Palo Alto, California, USA) system with ophthaldialdehyde (OPA) precolumn derivation, assembly system at 338 nm detection, a flow rate of 1.0 ml/min, and 40°C column temperature. Mobile phase A was (7.35mM sodium acetate/trimethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 with acetic

acid, while mobile phase B (pH 7.2) was (7.35mM) sodium acetate/methanol/acetonitrile (1:2:2, v: v: v). Amino acid composition was expressed as grams per 100gram of protein.

Enzyme Screening and Preparation of Protein Hydrolysate

The whole process to obtain protein hydrolysates and hydrolysate fractions has depicted in **(**Figure 1).The preparation of protein hydrolysate was done by the method previously reported (Ahn, Jeon, Kim, & Je, 2012) with a slight modification . Four kinds of proteases enzymes (Alcalase, Neutrase, Pepsin and Trypsin) were employed under optimal conditions (Table 1) for efficient enzyme screening in the preparation of dried laver protein hydrolysates. An enzyme/substrate ratio of 1:100 (w/w), 5% substrate for hydrolysis time of 4h was used. Freeze-dried protein powder of laver was mixed with appropriate buffer solution (1:20) adjusted by 1M hydrochloric acid (HCl) or sodium hydroxide (NaOH) to the required pH and then reacted with an enzyme at the recommended temperature for a period of hydrolysis time. Samples were taken in time intervals to determine the effective hydrolysis time. The pH was maintained for each hydrolysis process using either 1M NaOH or 1M HCl as appropriate, while the temperature was maintained using a temperature controlled thermostat water bath connected with the jacketed reactor. As soon as the enzymatic reaction was completed, laver protein hydrolysates (LPH) were boiled for 15 min at 95-100°C to inactivate the enzyme and supernatants were separated from the residue of non-hydrolysed and denatured proteins by using centrifuge (Avanti J-25, Beckman Coulter Inc.,

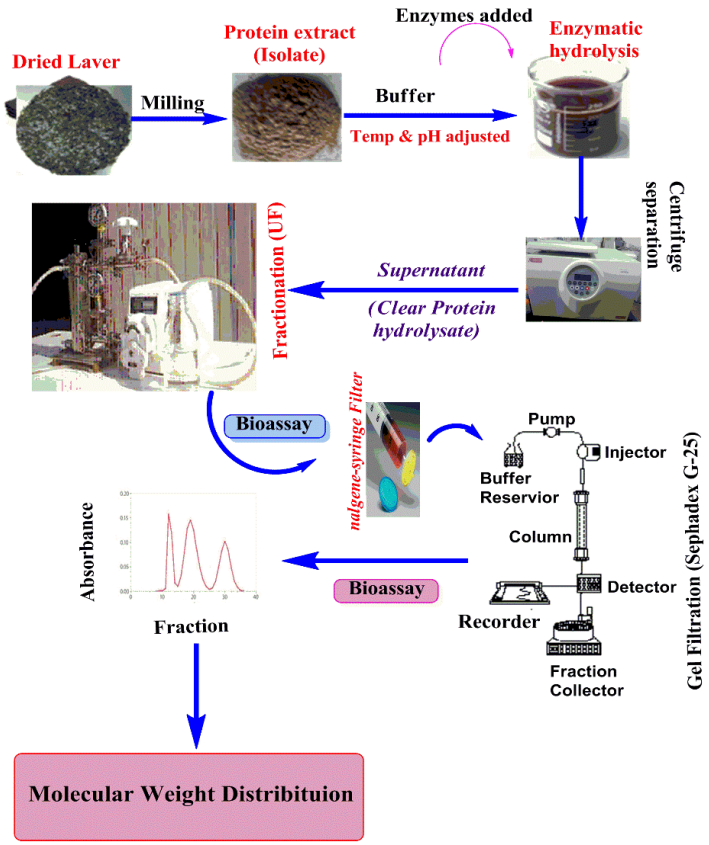

Figure 1. General schematics depicting production of protein hydrolysates and hydrolysate fractionations.

Table 1. Optimum conditions for commercial enzymes used in this study for hydrolysis of dried Laver protein

Enzyme	Optimum conditions		Buffer	Source
	pH	Temp(^{0}C)		
Pepsin	2.0	37	0.2M KB[1]	Porcine gastric mucosa
Trypsin	8.2	37	0.2M PB[2]	Porcine pancreases
Alcalase	8.0	50	0.2M PB	Bacillus Licheniformis
Neutrase	7.0	50	0.2M PB	Bacillus amyloliquefaciens

[1]Potassium chloride buffer, [2]Phosphate buffer

Fullerton, California, USA) at 10,000xg, 4°C for 20 min. The supernatant LPH was filtered through whatman® filter paper and analyzed to quantify α-amylase inhibition rate (α-AI rate). The most effective enzyme for achieving high α-AI rate was chosen.

α-Amylase Inhibition Activity (α-AI)

α-AI activity was assayed by measuring the residual α-amylase activity in the presence of the sample protein hydrolysate containing the inhibitor. The assay was performed by adding 50 to 200 µL of protein hydrolysate to 200µL of porcine pancreatic α-amylase solution (1U/ml) prepared with phosphate buffer (20mM, pH 6.9) containing sodium chloride (6.7mM), in order to obtain 50% inhibition. The control was prepared in the absence of the inhibitor with the addition of phosphate buffer (20mM, pH 6.9) containing sodium chloride (6.7mM) instead of a sample. A blank was run parallel without α-amylase in order to correct for any endogenous amylase activity. The mixtures were brought to a total volume of 400 µL with phosphate buffer (20mM, pH 6.9) containing sodium chloride (6.7mM) and incubated at 37°C for 20 min. After the addition of 200µL starch solution (10g/L) prepared in phosphate buffer (20mM, pH 6.9) containing sodium chloride (6.7mM) and incubated for 10 min at 37°C, the reaction was stopped by the addition of 400 µL Dinitrosalicylic acid (DNSA) color reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding distilled water, its absorbance was measured at 540 nm by UV-1100 spectrophotometer. The α-amylase inhibitory activity was expressed as percentage inhibition rate and calculated using the following formula:

$$Inhibition\ rate(\%) = \left[1 - \frac{Absorbance\ \ of\ sample}{Absorbance\ \ of\ control}\right] x100$$

Fractionation and Separation of Laver Protein Hydrolysates

LPH, were fractionated through an ultrafiltration (UF) membrane with molecular weight cut-off (MWCO) of 5 and 10kDa (Millipore Corp., Barnant co., Barrington, IL 60010, USA). Thus, permeate from the 5 kDa membrane (MW <5 kDa) was collected while the retentate was passed through the 10 kDa and both permeate (MW = 5-10 kDa) and retentate (MW >10 KDa) were collected separately. All fractions recovered were named as LPH-I (molecular weight <5 kDa), LPH-II (molecular weight = 5-10 KDa) and LPH-III (molecular weight >10 kDa). Finally, all the fractions recovered were determined for their α-amylase inhibition activity as described. The fraction with the highest α-amylase inhibition activity after UF membrane fractionation was loaded and separated using a Sephadex G-25 gel filtration chromatography column (1.0 × 100 cm). The column was pre-equilibrated with ultra-sonicated distilled water, and eluted with the same distilled water at a flow rate of 0.5 ml/min and monitored at 220 nm. The fractions with the desired peaks were pooled and lyophilized for α-amylase inhibition test and determination of molecular weight distribution.

Molecular Weight Distribution

The fraction with highest α-amylase inhibition activity from gel filtration was analyzed for their molecular weight distribution according to the procedure described by (Li, Jiang, Zhang, Mu, & Liu, 2008). A Waters TM 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA) was used. The hydrolysate was loaded onto TSK gel G2000 SWXL column (7.8 i.d. × 300 mm, Tosoh, Tokyo, Japan), eluted with 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 mL/min and monitored at 220 nm. A molecular weight calibration curve was obtained from the following standards from Sigma: cytochrome C (12,500 Da), aprotinin (6500 Da), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da). Results were processed using Millennium 32 Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

Statistical Analysis

All the experiments were made in triplicate and Duncan's multiple range tests by the SPSS software package (Version 17.0). $P \leq 0.05$ was used for a significant difference.

Results

Protein Extraction and Amino Acid Composition

In our current study, the crude protein content of dried laver powder was 42.99±0.05% DW and the protein content of its alkaline extract was 63.35 ± 0.08% (DW), the yield was 22.14±0.25%. The amino acid composition (g/100 g of total protein) of seaweed under study for the whole powder protein (WPP) and alkaline protein extract (APE) is presented in Table 2**.** Totally seventeen (17) amino acids were analyzed in this study for commercially dried laver. The analyzed amino acids represented both free and combined amino acids.

Preparation of Laver Protein Hydrolysate (LPH) and α-AI Activity

In this experiment, four proteolytic enzymes, namely, alcalase, pepsin, neutrase and trypsin were evaluated for their effectiveness on the degradation of commercial dried laver protein for α-amylase inhibitory activity. Figure 2A shows the hydrolysis

curve of inhibition rate of dried laver protein hydrolysates produced using the aforementioned commercial protease enzymes under their optimal conditions at different time intervals. Among all the enzyme hydrolysates, pepsin hydrolysate was found to be efficient in releasing α-amylase inhibitory peptides from the parent protein of commercial dried laver seaweed at the IC_{50} value of 1.86 mg/ml. The following α-amylase inhibitory were observed as: alcalase hydrolysate (31.73%), trypsin hydrolysate (26.42%), and neutrase hydrolysate (18.27%) in the same protein concentrate (1.86 mg/ml). A higher α-amylase enzyme inhibition rate or lower IC_{50} value indicates a stronger α-amylase inhibitory activity of the hydrolysate. The hydrolysis time in which each enzyme hydrolysate achieved higher α-amylase inhibition rate was different (Figure 2A). The results revealed that the hydrolysate produced by pepsin enzyme was statistically higher than that of alcalase, neutrase and trypsin (Figure 2B) ($p<0.05$). These results indicate that α- amylase enzyme inhibition activity of the LPH was affected by the type of proteolytic enzymes.

Table 2. Amino acids profile of whole powder protein (WPP), alkaline protein extract (APE) and the ratio of (APE/WPP) obtained from commercial dried laver (g/100g protein)

Amino acid	Letter Codes	WPP	APE	Ratio
Essential amino Acid (EAA)				
*Histidine	His	0.489	0.211	0.431
Isoleucine	Ile	1.617	2.768	1.712
Leucine	Lev	2.643	5.063	1.916
Lysine	Lys	1.684	2.985	1.773
valine	Val	2.407	4.328	1.798
Threonine	Thr	1.773	2.016	1.137
Phenyl alanine	Phe	1.566	2.301	1.469
Methionine	Met	0.632	1.198	1.896
Tryptophan	Trp	**nd	**nd	**nd
Non-essential Amino acids(NEAA)				
Arginine	Arg	2.099	4.169	1.986
Alanine	Ala	4.539	5.181	1.141
Aspartic acid	Asp	4.215	6.176	1.465
Glutamic acid	Glu	5.529	6.456	1.168
Glycine	Gly	2.352	3.245	1.380
Proline	Pro	1.435	2.012	1.402
Serine	Ser	1.204	2.649	2.200
Tyrosine	Tyr	0.783	2.368	3.024
Cysteine	Cys	0.029	0.073	2.517
Total and Percentage distribution of amino acid with different properties				
Acidic		9.744(27.845%)	12.632(23.745%)	1.296
Basic		4.272(12.208%)	7.365 (13.844%)	1.724
Hydrophobic		14.836 (42.397%)	22.851(42.955%)	1.540
Uncharged polar		6.141(17.5495)	10.351(19.457%)	1.686
Total amino acids g/100g protein		34.993	53.198	1.520

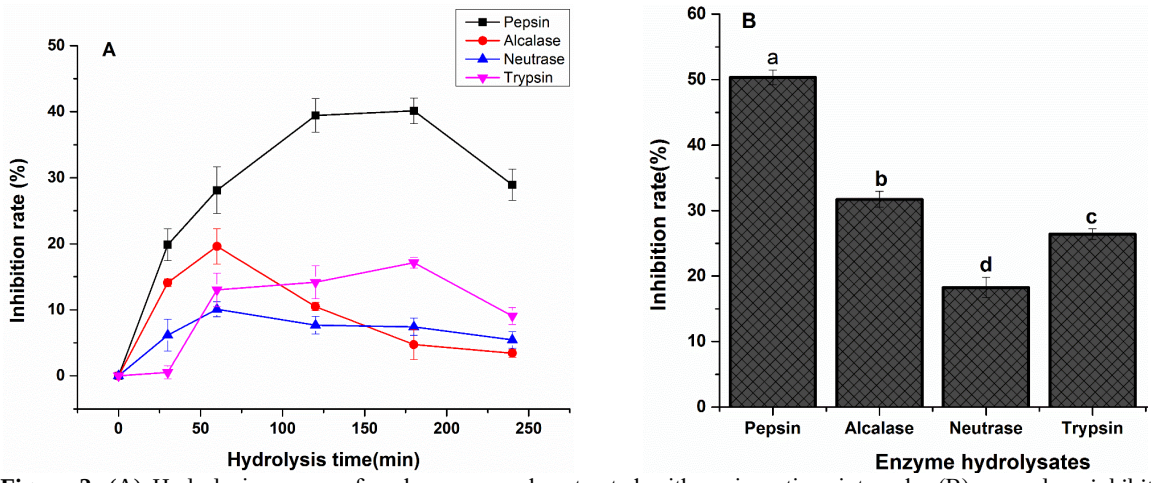

Figure 2. (A) Hydrolysis curves of each enzyme when treated with various time intervals, (B) α-amylase inhibition activity of each enzyme hydrolysate at 1.86 mg/mL protein concentration, Different letters (a, b, c ,d) indicate significance differences at $P \leq 0.05$.

Fractionation of α-Amylase Inhibitory Peptic Hydrolysate

The pepsin hydrolysate was fractionated into three MW groups, LPH-I (molecular weight <5 kDa), LPH-II (molecular weight = 5-10 KDa) and LPH-III (molecular weight >10 kDa) using UF membrane (Mwco = 10 kDa and 5 kDa). The three MW groups were investigated by the α-amylase inhibitory assay. As shown in Table 3, the molecular weight <5 kDa (LPH-1) fraction exhibited higher α-amylase inhibitory activity than other fractions and had an IC_{50} value of 1.18 mg/ml. This fraction (LPH-I) was subsequently loaded in to column (1.5 cm ×100 cm) chromatographic (size exclusion chromatography) method on a Sephadex G-25 gel chromatography. Three fractions were separated and designated as F-I, F-II and F-III (Figure 3A). As indicated in Figure 3B, the third fraction (F-III) possessed higher α-amylase inhibitory activity at IC_{50} value of 0.87mg/mL and followed by F-I. Fraction (F-II) exhibited the lowest α-amylase inhibition activity of all fractions.

Molecular Weight Distribution

Considering that F-III was found to possess the highest α-amylase inhibition activity, this fraction was therefore analyzed for molecular weight distribution (Figure 4). The chromatographic data indicated that this fraction was composed of low molecular weight peptides whose major peaks were located at 300-700 Da (47.28%) and 91-300 Da (66.41%).

Discussions

Protein data of marine algae presents many applications, involving both basic and applied research (Diniz, Barbarino, Oiano-Neto, Pacheco, & Lourenço, 2011). However, extraction is one of the main problems in seaweed protein analysis and extraction yields are generally low due to the presence of large amount of polyanionic cell wall mucilage's and phenolic compounds (Admassu, Zhao, Yang, Gasmalla, & Alsir, 2015; Diniz *et al*., 2011; Fleurence *et al.*, 1995; Wong & Cheung, 2001). After comparing with different classical and enzymatic procedures (eg, an aqueous polymer two-phase system, polysaccharidase, or Tris-HCL buffer), Fleurence *et al.* (1995) concluded that the highest yield of seaweed protein extract could be obtained by the use of sodium hydroxide (NaOH) and 2-mercaptoethanol after an initial aqueous extraction, in

Table 3 α-amylase inhibitory activity of UF fractions of Peptic hydrolysate

Fraction	IC_{50} value (mg/ml) [a]
Unfractionated [b]	1.86
> 10 kDa [C]	1.69
5 – 10 kDa [C]	1.54
< 5 kDa [C]	1.18
F-III [d]	0.87

[a] *The concentration of α-amylase inhibitory peptides required to inhibit 50% of the α-amylase enzyme activity,*
[b] *Pepsin hydrolysate(unfractionated),*
[c] *Fractions from UF membrane of molecular weight cut-off of 5 and 10 kDa was used,*
[d] *Sephadex G-25 gel chromatography*

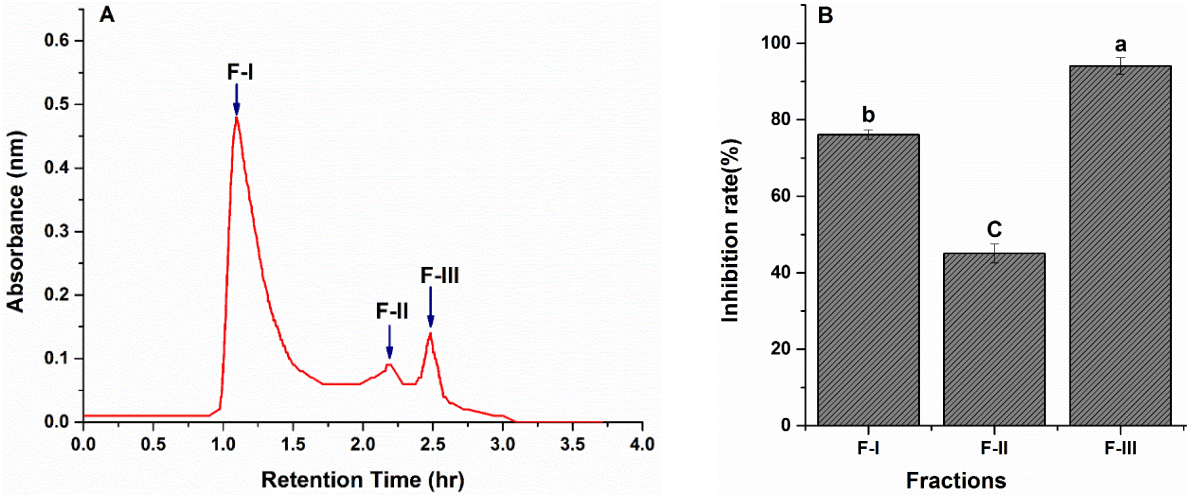

Figure 3. Gel chromatogram of UF Pepsin hydrolysate (MW<5 kDa): (A) separated peaks on Sephadex G-25 gel chromatography, (B) α-amylase enzyme inhibition activity of each fraction. Different letters (a, b, c) indicate significance differences at P≤0.05.

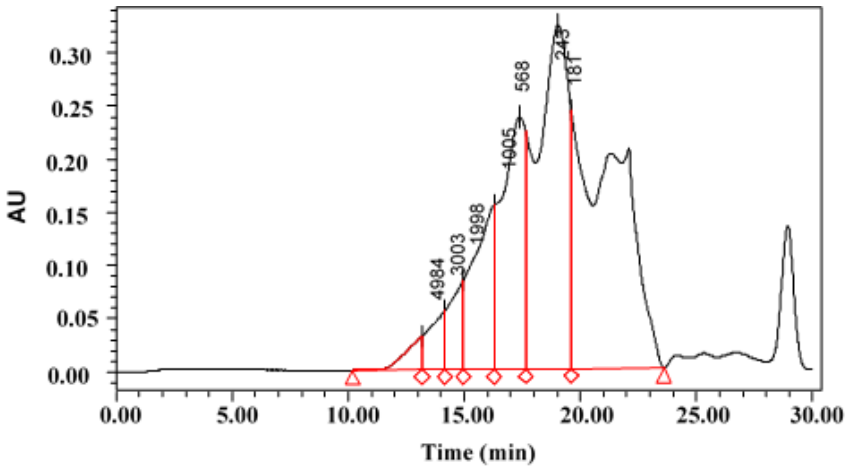

Figure 4. Molecular weight distribution of LPH fraction (F-III) having the highest α-amylase inhibition activity. Molecular weight range for major peaks: 300–700 Da and 91–300 Da.

which our present protein extraction from commercial dried laver was also done by using this method. Results found in our study for protein content was agreed with the previous report described by (Dawczynski, Schubert, & Jahreis, 2007; Fleurence, 1999a; Wong & Cheung, 2001) in that red and green seaweeds had total protein content within the wide range 10-47% (DW). The extraction of protein from the raw commercial laver has increased its content from 42.99±0.05% to 63.35±0.08%. This protein was used as a substrate raw material for enzymatic hydrolysis in this experiment.

Algae provide a significant amount of nitrogen compounds, namely, amino acids and proteins as well. The amino acid composition of seaweeds has been frequently studied and compared to that of other foods. For most seaweeds, an aspartic and glutamic acids constitute together a large part of the amino acid fraction (Fleurence, 1999b; Gressler *et al*., 2010). Previous experiments described that these two amino acids can represent between 22% and 44% of the total amino acids (Gressler *et al*., 2010). Acidic amino acids are typically predominant over basic amino acids in red seaweed (Cian, Alaiz, Vioque, & Drago, 2013). These amino acids are responsible for the special flavor of the seaweed (Hwang *et al.*, 2013). In our current study, glutamic acid was the most abundant amino acid in both WPP and APE (15.80% and 12.14% of the total amino acid respectively). Aspartic acid was the second most abundant amino acid in APE (11.61% of the total amino acid), however, alanine was the second in WPP (12.97% of the total amino acid). The percentage of cysteine was the lowest of all amino acids in both WPP and APE (0.08% and 0.14% of the total amino acid respectively).

One fundamental consideration is that algae (Micro or macro algae) are the base of the aquatic food chains, which can produce the food resources that fish are adapted to consume. A critical shortcoming of the crop plant proteins commonly used in fish feeds is that they are deficient in certain amino acids such as lysine, methionine, threonine, and tryptophan. Various studies on the relationship between the nutritive value of feeds and culture organisms have demonstrated that the content of essential amino acids and fatty acids is the principal factor in their dietary value. Many marine animals have a limited ability to synthesize essential amino acids as well as the unsaturated fatty acids of the omega-3 series, however, these substances are essential dietary nutrients for good growth and survival of fish. Marine macro algae could be a potential low-cost source of protein for fish feed (Vinoj & Kaladharan, 2007). The analysis of the amino acid content of algae have found although there is significant variation due to species or season in their concentration , they generally contain all the essential amino acids (Dawczynski *et al*., 2007, Harnedy & FitzGerald, 2011). Commercial dried laver is one of the potential resources that could contribute for fish feed as it contains all essential amino acids.

In this study, except for tryptophan, all essential amino acids were determined. The essential amino acids (EAA) amounted to 12.81g/16g nitrogen for WPP and 20.87g/16g nitrogen for APE, which was less than for most red and brown seaweed varieties and the ratio of EAA/non-essential AA of WPP and APE (0.58:1& 0.65 :1 respectively), found to be less than the previous research results reported by Dawczynski *et al.*, (2007) for *Porphyra* species from Japan and Korea and China (0.7:1&0.8:1 respectively). On average, extraction of protein increased the total amino acid composition by a factor of 1.5 of the raw powder of dried laver.

Bioactive peptides are often functionally inactive within the native proteins structure and must be released from the original protein after degradation to fulfil their specific bioactive roles (Udenigwe,

2014). These bioactive peptides can be generated by hydrolytic reactions using various protease enzymes. Selection of appropriate hydrolytic enzyme or an optimal mixture of enzymes is vital to obtain expected outputs (Jeon & Wijesinghe, 2012). The type of bioactive peptides generated from a particular protein is dependent on two factors: (a) the primary sequence of the protein substrate and (b) the specificity of the enzyme(s) used (Harnedy & FitzGerald, 2013). Among the employed proteolytic enzymes to hydrolyse dried laver seaweed protein in our study, pepsin hydrolysate showed effective α-amylase inhibition. Peptic hydrolysates may contain materials that could react with α-amylase enzyme active sites that compete with the starch substrate during hydrolysis. Several studies have suggested that the activity of peptides in hydrolysates varies depending on their amino acid sequence and length of peptides (Kim *et al.*, 2001). This suggests that enzymatic hydrolysis with different proteolytic enzymes would lead to the formation of different peptide sequences. Pepsin, an aspartic protease, mainly acts on the N-terminal aromatic amino acids, and several studies have suggested that pepsin is useful for producing bioactive peptides from fish protein such as tuna dark muscle, hoki frame protein, and smooth hound muscle protein (Bougatef *et al.*, 2009). Other reports indicate that pepsin is capable of producing ACE inhibitory peptides (Sheih, Fang, *et al.*, 2009; Suetsuna & Maekawa, 2004; Suetsuna & Nakano, 2000) and antioxidant peptides from algae and fish proteins (Ahn, Je, & Cho, 2012; Ahn, Kim, & Je, 2014; Je, Qian, Byun, & Kim, 2007; Sheih, Fang, *et al.*, 2009; Sheih, Wu, & Fang, 2009). Considering in achieving a high α-AI activity of peptic hydrolysate, compared to that of other proteolytic hydrolysates produced in this study, we selected pepsin as an effective enzyme to hydrolyse laver protein, and pepsin hydrolysate was then chosen for further purification and fractionation studies.

Ultrafiltration (UF) membrane technology has been successfully utilized to obtain powerful protein hydrolysate fractions, which could avoid the loss of bioactive substances during treatment (Ko *et al.*, 2012; Wu, Jiang, Jing, Zheng, & Yan, 2016). In view of this, in order to obtain fractions with better α-AI activity, a peptic hydrolysate of commercial dried laver protein fractionated with UF membrane in to different molecular weight cut-offs. In this experiment, the <5 kDa molecular weight fractions showed a better inhibition rate. It has been previously reported (Ko *et al.*, 2012) that a marine *Chlorella ellipsoidea* hydrolysate was fractionated into three fractions (>10, 5-10, and <5 kDa) using UF and the <5 kDa fraction exhibited strongest ACE inhibition activity and had an IC_{50} value of 0.89±0.04 mg/mL .It is possible to understand that low molecular weight fractions had more potent bioactivity than that of the high molecular weight fractions. Gel filtration chromatography further separates and purifies the hydrolysates and gives efficient activity. Based on this, the effective fraction from UF membrane has been subjected to gel filtration and fractionated in to three fractions (F-I, F-II, F-III). F-III was showing highest inhibition rate and studied for its molecular weight distribution. A number of studies had already shown that the biofunctional activity of hydrolysates is dependent on their molecular weight distribution (Moure, Sineiro, Domínguez, & Parajó, 2006; Peña-Ramos, Xiong, & Arteaga, 2004). In this study, results revealed that the peptide fraction with a molecular weight ranging from 90 to 700 Da was associated with a higher α-amylase inhibitory activity. These findings are in agreement with observations from other studies and support the fact that functional properties of peptides are highly influenced by properties such as molecular mass (Dong *et al.*, 2016).

Conclusions

In this study, we have evaluated the α-amylase inhibitory potential of enzymatically hydrolysed commercial dried laver protein. Using consecutive fractionation, the hydrolysate fractions exhibited a promising highest α-amylase inhibitory activity depending on their molecular cut off, showed that the smallest molecular weights are bioactive than larger molecular weight compounds. In general, the results of this study, suggested that commercial dried laver seaweed *(Porphyra* species*)* could be used as food supplement or pharmaceutical industrial applications in diabetes management.

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