EVALUATION OF BIOACTIVE PEPTIDES FROM *Gracilaria changii* PROTEIN

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DECEMBER, 2020

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December, 2020

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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LIST OF ABBREVIATIONS

ACE	-	Angiotensin-I-Converting Enzyme
DPP IV	-	Dipeptidylpeptiase –IV
DPP III		Dipeptidylpeptiase –III
E/S	-	Enzyme to substrate ratio
GLP-1	-	Glucagon –Like-Peptide 1
DM2	-	Diabetes Mellitus 2
GIP	-	Glucose- dependent Insulinotrophic Polypeptide
EU	-	Experimental unit
HCL	-	Hydrochloric acid
H_2SO_4	-	Sulphuric acid
EC_{50}	-	Half maximal inhibitory concentration
mg/g	-	Milligram per gram
mg/mL	-	Milligram per millilitre
mM	-	MilliMolar
µM	-	MicroMolar
mmHg		Millimeter of mercury
mU/mL	-	MilliUnit per millilitre
MWCO	-	Molecular Weight Cut Off
NaOH	-	Sodium Hydroxide
SDS		Sodium Dodecyl Sulfate
NO	-	Nitric oxide
RAS	-	Renin-Angiotensin System
KDa	-	Kilodalton
%	-	Percentage
&	-	And
et al	-	Et alii/et aliae/et alia

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ABSTRACT

Gracilaria changii is an agarophytic red seaweed mostly found in mangroves. Previous resarchers indicated that *Gracilaria changii* possess high protein(12.57 % of dry weight). So, its proteins are potential precursors for relasing bioactive peptides. To date, no study has been reported on the evaluation of *Gracilaria changii* proteins as a precursors of bioactive peptides. In the present study, bioactive peptides in phycocyanin alpha subunit(protein) of *Gracilaria changii* was evaluated based on in silico approach. In silico analysis of phycocyanin alpha subunit exhibited in high numbers of bioactive peptides predominately with dipeptidylpeptiase -IV(DPP-IV) inhibitory peptides and angiotensin-I-converting enzyme (ACE-I) inhibitory peptides having lower EC_{50} value. Other bioactive peptides are DPP III inhibitor, antioxidative, Renin inhibitor , alpha glucosidase inhibitor and neuropeptide. The application of silico tools provided rapid identification of protein. Overall, this study highlighted the potentiality of phycocyanin alpha subunit as a raw ingredient for developing pharmaceuticals products or functional foods.

Keywords: *Gracilaria changii*, bioactive peptides, in silico, DPP-IV inhibitor, ACE inhibitor.

CHAPTER 1: INTRODUCTION

Macroalgae, generally known as seaweed are large colonies of diversified algae that grows in both marine ecosystems and freshwater over the world (Moss & Mcsweeney, 2021). Macroalgae are a potential source of various invaluable macro and micronutrients, carrying proteins, carbohydrates, phenols, vitamins, and minerals (Lafarga *et al*., 2020).

Bio-active peptides are defined as peptide sequences within a protein that exerts a beneficial effect on body functions, beyond its known nutritional value (Kitts and Weiler, 2003). Bioactive peptides such as antihypertensive (Wijesekara and Kim, 2010) and anti-diabetic peptides (Wang *et al*., 2015) are widely observed due to their good potential as pharmaceutical products, particularly for human health enhancement. Hypertension is one of the major risk factors causing cardiovascular diseases and generally occurs with obesity, pre-diabetes, and atherosclerosis (Gouda *et a*l., 2006). The degradation of angiotensin -I and bradykinin by the angiotensin-I converting enzyme (ACE-I) within the renin-angiotensin- aldosterone system (RAAS) stimulates the increase of blood pressure and leads to hypertension (Weir & Dzau, 1999). Dipeptidyl peptidase IV (DPP-IV) cleaves incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent- insulinotropic peptide (GIP), resulting in Diabetes Melitus-2 (Singh *et al*., 2017). Though some synthetic therapeutic drugs have been generated to treat hypertension and Diabetes Melitus -2 (DM2); the majority of them are regarded as unsafe due to the side effects associated with their consumption. These include inflammatory responses, taste disturbance, nausea, headache, and allergic reaction (Singh *et al.*, 2017; Vigersky, 2006). Furthermore, bioactive peptides derived from natural sources like

marine fish and dairy products, concerted with drugs as supportive agents have been considered as an alternative treatment for the disease (Panjaitan *et al.,* 2018).

In silico approaches, as a supplement to empirical methodologies, are capable of assessing the potential of proteins as precursors of bioactive peptides and predicting the specific activities of some peptide sequences (Udenigwe, 2014). Additionally, it is frugal and more time-saving than experimental investigations (Li-Chan, 2015). The BIOPEP, a database of bioactive peptide fragments, helps predict the potential bioactivity of peptides and their corresponding activities (Dziuba *et al*., 2009).

1.1 Problem Statement

Gracilaria is the second largest genus of red algae comprising more than 150 species distributed worldwide (Yow *et al*., 2011). Red seaweed has higher protein than green and brown seaweed (Fleurence, 1999). Thus, its protein specifically phycocyanin may be a potential substrate to release bioactive peptides with ACE inhibitory activity and DPP-IV inhibitory activity. Besides that, the use of synthetic ACE inhibitors poses many side effects to the human body such as cough, cancer, and rash. Furthermore, nowadays, more consumers are health conscious and they prefer natural ingredients for functional foods compared to synthetic chemical products. To the best of our knowledge, no study has been reported on the potential of *Gracilaria changii* proteins as a precursor of bioactive peptides.

1.2 Significance of Study

This study will generate new knowledge on natural and safe DPP -IV and ACE inhibitory activity from *Gracilaria changii* protein. This study will help pharmaceuticals industry to find out a raw ingredient for generating pharmaceuticals products. This study may give an alternative source to the health-conscious consumer for natural food-based products, as they are safe and also environmentally friendly (Veeresham, 2014). This study may also be a guideline to the food industry for developing nutraceutical and value-added products. Furthermore, value added products from seaweed species may also increase the economic value of seaweed industry.

1.3 Objective

1. To evaluate the bio-active peptides from the *Gracilaria changii* protein extract.

2. To determine the half maximal inhibitory concentration (EC_{50}) of ACE inhibitory activities and DPP-IV Inhibitory activities from *Gracilaria changii* protein.

3. Rapid identification of protein from *Gracilaria changii* using bioinformatics (In silico) tools.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Seaweed

2.1.1 Characteristics of seaweed

Seaweed is a member of the algal group that comprises about 10,000 species. According to previous publications, seaweed for human usage is generated from China 1700 years ago (Yang *et al*., 2017). It is also known as "marine algae" which form dense forests in coastal water. Oscillation in temperature, light, osmotic stress and desiccation is the brutal environment confronted by the seaweed (Gupta and Abu-Ghannam, 2011). It varies in size ranges from the smallest microscopic single cells to the largest plants, for example, giant seaweeds (Lee, 1987; Raj, 2018).

Seaweed is of ecological importance that contributes around 10% of the total world marine productivity and acts as an important primary producer in the food chain which supplies oxygen to the sea. It serves not only as food and production of hydrocolloid but also as an ingredient in cosmetics and fertilizers (Chan *et al*., 2006).

Seaweed can classify into three main phyla based on pigmentation which are Chlorophyta (green), Rhodophyta (red), and Phaeophyta (brown). The green pigment of Chlorophytes is due to the dominance of chlorophyll as in land plants while the brown pigments of Phaeophytes are due to the presence of fucoxanthin and xanthophylls. Rhodophytes, the red algae seaweed has red color because of the phycoerythrin pigment (Raj, 2018). Besides red pigment, Rhodophytes also have blue pigments such as phycocyanin and phycoerythrin. The red and blue pigment give benefit to Rhodophytes as it can absorb blue-green light in the sea for food production by photosynthesis (Pal *et al*., 2014).

2.1.2 Nutritional composition of seaweed

The protein content in seaweed is generally high especially in Rhodophyta or red seaweed (Mohamed *et al*., 2012). Table 2.1 shows the general nutritional composition of seaweed (Rohani-Ghadikolaei *et al*., 2012).

Table 2.1: Nutritional composition of seaweed (Rohani-Ghadikolaei *et al*., 2012)

Composition	Moisture	Lipid	Protein	Ash	Carbohydrate
Dry weight basis (%)	6 – 12	1 - 5	10 - 30	12 - 30	30 – 60

2.1.3 Applications of seaweed

The protein content in seaweed is generally high especially in Rhodophyta or red seaweed (Mohamed *et al*., 2012). Seaweed as a source of food, agar, and gelling substance is valuable for society. At present, seaweed is not only used in food and pharmaceutical industries for economical purposes, but also acts as a growth medium for bacteriological studies (Pal *et al*., 2014). Traditionally, seaweed has been used as the main food source for more than 14,000 years, especially in Pacific and Asian countries (Gofii *et al*., 2000). Seaweed has a high nutritional value such as protein, carbohydrate, minerals, dietary fiber, and vitamins with low calories content which make it potential as a portion of healthy food (Baptista *et al*., 2011). Table 2.2 shows the different applications of seaweed.

Table 2.2: Applications of seaweed

Application	Reference
Food source such as bread, condiments, drinks, noodles, salad, soup, sushi	(Bouga and Combet, 2015)
Animal feed and supplement especially apply for fish	(Schuenhoff *et al*., 2003)
Growth medium for bacteriological studies	(Pal *et al*., 2014)
Restoration ecology	(Haapalehto *et al*., 2017)
Ingredient in the field of pharmacology	(Raj, 2018)

2.1.3 *Gracilaria changii*

Gracilaria is one of the largest genera of red algal phylum Rhodophyta with more than 100 species distributed worldwide at intertidal and sub-tidal zones (Gulbransen *et al*., 2012). Gracilaria genus is an essential marine seaweed to humans nowadays because it is a worldwide source of agar (Marinho and Bourret, 2005). It also serves as fresh vegetables *Gracilaria changii* is a type of red seaweed that is abundant in Pattani, Thailand (Benjama and Masniyom, 2011).

2.1.4 Taxonomy and Morphology

The colour of *G. changii* is dark red while the thallus could grow between 180 mm to 220 mm tall. Primary branches are shorter compare to secondary branches and reach up about 25 mm to 40 mm long while secondary branches reach up around 40 mm to 170 mm. The species discoidal holdfast and the branches are irregular with diameter between 1 to 2 mm. Constriction occur at base of branches, swelling at middle and tapering toward the end. The formation of branches occurs occasionally. The tip of secondary branches either are divided into two short branchlets. Formation of new branches with pointed tip are seen along tertiary branches. The cross section of stipe stipe is composed of 3–4 layers of parenchymatous cells and surround by 2–3 layer of small rounded cortical cells at the cortex (Nur *et al*., 2018).The taxonomy classification of *Gracilaria changii* is shown in Table 2.3.

Table 2.3: Taxonomy classification of seaweed (*Gracilaria changii*) (I.A.Abbott., 1999)

Rank	Name
Kingdom	Plantae

Phylum	Rhodophyta
Class	Rhodophyceae
Subclass	Florideophyceae
Order	Gracilariales
Family	Gracilariaceae
Genus	*Gracilaria*
Species	*Gracilaria changii*

2.1.5 Nutritional composition of *Gracilaria changii*

Several studies have been reported on the nutritional composition of *G. changii*. Freeze-dried *Gracilaria changii* contents high dietary fibre (64.74 ± 0.82%), low fat (0.30 ± 0.02%) and Na/K ratio (0.12 ± 0.02). The total amino acid content (91.90 ± 7.70%) is mainly essential amino acids (55.87 ± 2.15 mg g-1) (P. T. Chan & Matanjun, 2017). The red algae *G. changii* contain (wet weight basis): total protein (6.9±0.1)%, crude fiber, (24.70±.7)%; total lipid, (3.30±.2)% and ash, (22.7 ±0.6%.)(Norziah and Ching, 2000).

2.1.6 Previous study on *Gracilaria changii*

Besides the nutritional composition of *G. changii*, there are limited studies reported on *G. changi*i as shown in Table 2.4. Sasidharan *et al.* (2008) reported that the methanol extract of *Gracilaria changii* exhibited antimicrobial activity for in -vivo brine shrimp lethality and in vitro anticancer cell line activity. Badranei *et al*, (2020) have reported on seaweed *G. cangii* as effective bioremediation for shrimp P. vannamei culture because seaweed reduces ammonia that is toxic for shrimp.

Table 2.4 Previous study on *Gracilaria changii*

Previous findings	Reference
Study on *Gracilaria changii* to evaluate toxicity	(Sasidharan *et al.*, 2008)
Genetic diversity of *Gracilaria changii* (Gracilariaceae, Rhodophyta) from the west coast, Peninsular Malaysia based on mitochondrial cox-1 gene analysis	(Yow *et al.*, 2011)
Determination of antioxidant and hypolipidaemic properties of red seaweed, *Gracilaria changii*	(P. T. Chan *et al.*, 2014)
Seaweed *Gracilaria changii* as a bioremediaton agent for ammonia, nitrite and nitrate in controlled tanks of Whiteleg Shrimp *Litopen aeusvannamei*	(Badraeni *et al.*, 2020)

2.2 Seaweed Drying

Researchers reported that after cleaning red seaweed, clean seaweed is immediately placed in a freezer(-40°C) and then freeze-dried in a freeze–dryer for 24 h.(P. T. Chan *et al.*, 2014). Biomass drying methods such as freeze, vacuum, solar and convective drying play an important role in protein yield from seaweeds along with affecting their functional properties (Abdullah, 2019). Many researchers have reported that Freeze-drying prior to protein extraction resulted in higher protein content as well as antioxidant activity in five species of brown seaweeds (*Fucus spiralis, Laminaria digitata, Fucus serratus, Halidrys siliquosa, Pelvetia canaliculata)*. But the green seaweed, Ulva sp., a convective drying method (hot air at 70°C, airflow rate of 2.0 m/s for 120 min) provide higher crude protein yield (20%) compared to freeze, vacuum or solar-drying Moreover, the biomass that procures from a convective drying holds higher antioxidant activity. (Cermeno *et al.*, 2020)

2.3 Protein extraction from seaweed

For plant-based protein, protein extraction is needed prior to protein enzymatic hydrolysis because the protein is entrapped in the cell wall of the seaweed. Extraction is defined as a separation technique of the substances in a mixture by dissolving the test component in solvents that yields at least two components which are the solute or extracted solution and the residue (Benaiges and Guillen, 2007). Raw and unprocessed seaweed has poor protein digestibility, and thus tend to reduce the bioactivity of seaweed. The development of different methods for seaweed protein extraction has helped to improve and increase bioavailability (Fleurence *et al*., 2004).

The efficiency of extraction is influenced by two main factors : a) Chemical composition of the seaweed species , b) Morphological or structural characteristics of seaweed (Barbarino and Lourenço, 2005). Red, green and brown seaweeds have significant differences in terms of chemical composition including protein, fiber, carbohydrate, lipid and moisture content. This makes the seaweeds to pose different ability in the formation of a variety of products (Ciko *et al*., 2018).

The multiple layers cell wall of seaweed is not only formed by the combination of polysaccharides, alginates and carrageenan but also associate with various interactions of bonds including calcium and potassium (Wijesinghe and Jeon, 2013). Successful extraction of protein in seaweed is influenced by the availability of the protein molecules in seaweed. But the protein in seaweed is hindered by anionic cell-wall polysaccharides including the carrageenan in red seaweed and alginates in brown seaweed. So, an enzyme such as polysaccharidase is used for cell disruption as a part of pre-treatment. This pre-treatment is applied to increase protein yield during protein extraction (Bleakley and Hayes, 2017). Table 2.5 shows the different chemical extraction methods for precipitating proteins from seaweed.

Table 2.5: Different types of extraction methods for precipitating proteins from seaweed

Seaweed used	Type of extraction	Protein yield on dry basis	Reference

Irish brown seaweed (*Ascophyllum nodosum*)	Treatment with acid (HCl) and alkali (NaOH)	59.76% yield	(Kadam *et al*., 2017)
	Sequential extraction	59.76% protein extracted had a mean mass of 3.27 kDa	
	Ultrasound pre-treatment	Increase protein extraction with acid and alkaline treatment alone by 540% and 27%, respectively and time interval from 60 min to 10 min	
Ulva rigida* and *Ulva rotundata	Deionized water	Low macromolecule protein yield for each species	(Fleurencel *et al*., 1995)
	Tris HCI (0.1 M) buffer	-	
	Extraction with Tris HCI (0.1 M) buffer with sonication	-	
	The first extraction with Tris HCI (0.1 M) buffer and second extraction with NaOH (0.1 M)	Remarkably improves the yield	
	First extraction with deionized water and second extraction with NaOH (0.1 M)	Highest protein yield	
	Extraction in the aqueous polymer two-phase system	Notably increases the recovery of proteins (2.0 fold for *U. rigida*, 2.3 fold for *U. rotundata*)	
Algal cultures (*Scenedesmus, Svnechococcus, Asterionella*)	Standard method extraction	-	(Rausch, 1981)
Palmaria palmata	Polysaccharidase	-	(Joubert and

	degradation (enzymatic hydrolysis)		Fleurence, 2007)
Gelidium pusillum	Aqueous two-phase extraction	-	(Mittal *et al.*, 2019)
Palmaria palmata	Osmotic stress	thirty-ninth percent protein yield	(Harnedy and FitzGerald, 2013)
	High shear force	40% protein yield	
	Alkaline and aqueous	24% protein yield	
Brown seaweed (*Macrocystis pyrifera*) and red seaweed (*Chondracanthus chamissoi*)	Enzyme assisted extraction (EAE)	*M. pyrifera*: 74.6% protein yield *C. chamissoi:* 36.1% protein yield	(Vasquez *et al*, 2019)
***Ulva* sp.**	Deionized water and treatment with β-mercaptoethanol	20% protein yield	(Kazir *et al.*, 2019)
	Deionized water without β-mercaptoethanol	30% protein yield	
	Deionized water and ultrasonic bath	20% protein yield	
	Lysis buffer	30% protein yield	
	NaOH and ultrasonic bath	65% protein yield	
Porphyra umbilicalis, Ulva lactuca,* and *Saccharina latissima	Sonication in water and ammonium sulphate salt precipitation of protein	Greatest protein yield once applied to *U. lactuca* (19.6 ± 0.8%)	(Harrysson *et a*l., 2018)
	Accelerated solvent extraction		
	pH-shift method	*P. umbilicalis* (22.6 ± 7.3%) and *S. latissima*(25.1 ± 0.9%)	

Brown seaweed (*Saccharina latissima*)	pH-shift method	Total protein yield does not exceed 11.2%	(Abdollahi *et al*., 2019)
Eucheuma cottonii	Phenol/lysis buffer Extraction	0.027 ± 0.000 mg/g) protein yield	(Lim and Teo, 2015)
	Phenol (TRI reagent)/ chloroform extraction	0.018 ± 0.001 mg/g protein yield	
	Phenol/SDS Buffer Extraction	0.024 ± 0.002 mg/g protein yield	

Ammonium sulphate is the most traditional and most commonly used extraction method for salting out proteins because it is a cheaper and safer chemical reagent. The basic theory to extract protein from plants is generally based on the solubility of globular proteins in the plant. Ammonium sulphate is a type of salt where can increase the solubility of globular protein when the addition of salt at less than 0.15 M. This process is defined as salting-in. The protein solubility decreases at higher ammonium sulphate concentrations and thus lead to precipitation of protein (Green and Hughes, 1955). Ammonium sulphate is the commonly used extraction method for plants as it poses several advantages. The protein extraction through ammonium sulphate is reported to be faster, easier and simple when compare to other extraction methods (Oh *et al.,* 2013).

2.4 MALDI-TOF Mass Spectrometry

MALDI was introduced in 1988 by Karas and Hillenkamp. It is supported the employment of organic matrices powerfully gripping the wavelength of the optical maser used (often a pulsed nitrogen laser at 337nm) to desorb and ionize in a very comparatively soft manner intact Mass compounds. MALDI is a discontinuous particle production technique that is, of course coupled to discontinuous mass analyzers. TOF mass spectrometers square measure presumably the best mass analyzers by principle, and square measure ideally fitted to MALDI particle sources. The power of the MALDI technique in analyzing completely different categories of biomolecules like peptides, proteins, polysaccharides, polynucleotides (Chaurand *et al.*, 1999). Previous studies showed that amide sequences were foreseen by matrix-assisted optical maser desorption/ionization-time of flight bike mass spectrographic analysis (MALDI-TOF/MS) technique employing a 4700 genetics instrument with Denovo soul computer code (Applied Biosystems, Carlsbad, CA, USA). Researchers also noteded that MALDI-TOF may be a key analytical technique in macromolecule chemistry. It is the most popular technique for the identification of macromolecules (Jurinke *et al.*, 2004)

2.5 Protein hydrolysis

Protein hydrolysates are the breakdown product of protein into protein fractions which include the mixtures of polypeptides, oligopeptides and amino acids through partial hydrolysis (Schaafsma, 2009). Marine protein hydrolysates can be prepared by

proteolytic food grade enzymatic process, simulated gastrointestinal digestion, fermentation process and solvent extraction (Vijaykrishnaraj and Prabhasankar, 2015). Enzymatic protein hydrolysis is generally composed of the nucleophilic attack of a water molecule, which catalysed by a peptidase, on the covalent peptide bond between the carboxyl and amino groups of two adjacent amino acids (Wouters *et al*., 2016). There are two categories in terms of protein fractions based on different characteristics. Firstly, the category consists of protein fractions with a high amino acid content. Secondly, the category consists of inactive bioactive peptides with an amino acid sequence (Thiquynhhoa *et al*., 2015). The efficiency and functionality of a protein hydrolysate highly depend on the molecular size, structure and amino acid sequences of peptide generated (Chabanon *et al*., 2007).

Over the last two decades, the preparations of bioactive peptides have continually been discovered because the short chain peptides from proteins hydrolysis have a higher nutritive value and can be further utilized more efficiently than an equivalent mixture of free amino acids (Kaminski *et al*., 1986). Previous studies have shown that the protein hydrolysates derived from marine products such as fish, mollusk, bivalves, seaweed, possessed numerous fundamental metabolic processes (Mohamed *et al*., 2012; Millan-Linares *et al*., 2014;Lee *et al*., 2015). Protein hydrolysis involved the use of proteases from animal such as chymotrypsin, trypsin and pepsin, plants and microorganisms. The use of mild temperatures and pH levels in enzymatic protein hydrolysis makes the nutritional properties of the protein hydrolysates remain almost unchanged which are regarded safe for human nutrition (McCarthy *et al*., 2013).

Protein hydrolysis is commonly used in food industry to improve the functional and physical properties of proteins such as solubility, emulsification, gelation, water-holding capacities, fat-holding capacities and foaming ability in various types of foods (Guan *et al*., 2006). In addition, biopeptides also offer several advantages that make them preferable in pharmaceutical and food industries, due to the presence of wide spectrum of therapeutic action, low levels of toxicity and structural diversity in the product with different length and amino acid and sequences (Nasri, 2017).

2.5.1 Preparation of protein hydrolysates

According to Mandawat (2016), the preparation of protein hydrolysates can be classified into three stages, which are sample preparation, hydrolysis and purification. The first stage is sample preparation. In most situation, the main purpose of sample preparation is to both remove interferences and to pre-concentrate the analytes into a phase that suitable for the selected final analysis (Bergstrom, 2006). After the washing process, pre-treatment follow by dehydration using air drying method (Paiva *et al*., 2017) or other drying method and size reducing depend on the requirement of the analysis. For animal protein, the protein can be hydrolysed straight away without any extraction. However, for plants protein, protein extraction is needed prior to hydrolysis .

The second stage of preparation of protein hydrolysates is enzymatic hydrolysis. The protein is mixed with a buffer solution. Then, the selected enzyme is added homogeneously into the seaweed buffer solution. The major enzyme groups used for protein hydrolysate production are bacterial proteases such as Alcalase and Protamex (Bleakley and Hayes, 2017). Besides that, gastrointestinal proteases such as pepsin and

plant origin such as bromelain (Zhao *et al*., 2009) and papain (Kittiphattanabawon *et al*., 2013) also have been reported to use in production of protein hydrolysates.

The variables such as temperature, time, pH and E/S are adjusted to the optimum condition. Protein hydrolysis take places under mild processing conditions of pH 6 to 8 and temperature 40 to 60°C, which can minimizes side reactions (Hernandez-Ledesma *et al*., 2011). During the hydrolysis process, the degree of hydrolysis (DH) or bioactivity or functional properties of protein can be chosen as specific indicator. DH is a measurement of the extent of hydrolysis degradation of a protein (Nedjar-Arroume *et al*., 2008) and can be defined as the percent ratio between the number of peptide bonds cleaved and the total number of bonds available for proteolytic hydrolysis (Sbroggio *et al*., 2016). However, longer hydrolysis time may cause the peptides lost their ability such as the ability to inhibit ACE (Wu *et al*., 2008). Enzymatic hydrolysis is terminated by deactivating the enzyme at high temperature, commonly 90°C for a period of time. The mixture is allowed to cool down and is known as protein hydrolysate.

The final stage is purification. Heat inactivation, ultrafiltration, hydrolysis by exo-proteases and treatment with specific enzymes are the most common process in post-hydrolysis. Table 2.6 shows the main post-hydrolysis processes and the function of processes (McCarthy et al., 2013, Manadawat, 2016). Centrifugation or filtration is performed on the hydrolysis mixture containing the peptides as well as the unhydrolyzed debris and other residues (Vissesangua and Benjakul, 2012). The supernatant or precipitate is then dried through different drying method, for instance, freeze drying, oven drying and spray drying. Among the drying methods, freeze drying at - 80°C is the

most preferable method since it minimises physical damage, oxidation and thermal reactions and preserves the characteristic chemical composition of the seaweeds (Wong and Cheung,2001). The powder formed protein hydrolysates can give benefit applications during the analysis such as reduced in volume, require less storage space and increase shelf life (Cano-Chauca *et al*., 2005).

Table 2.6 : Post-hydrolysis processes and the function of each processes (Mandawat, 2016)

Process	Function
Heat inactivation	Inactivation of proteolytic enzymes
Use of specific enzymes	Reduce the content of specific amino acids
Ultrafiltration	Removal of high molecular weight proteins and peptides
Activated carbon	Reduction of bitterness
Hydrolysis by exo-proteases	Hydrolysis, reduction of bitterness
Absorption chromatography	Reduce the content of aromatic amino acids

Besides that, the functional properties of the native protein could be enhanced through enzymatic protein hydrolysis without decreasing its nutritional value because proteolysis allows the activation of amino acids in the peptides that are encrypted in the protein structure. The peptides produced remain highly soluble under both acidic conditions and thermal treatments. Protein hydrolysates produced through enzymatic protein hydrolysis can have better organoleptic and sensory properties (Betancur-Ancona *et al*., 2009). In addition, enzymatic protein hydrolysis not only increases the amount of hydrophilic and polar groups but also reduces the molecular weight of peptides. This results in changes of the structure of protein globular structure since the hydrophobic regions of the protein are hidden and directly influence the emulsifying and foaming properties of protein hydrolysate (Van der Ven *et al*., 2001).

Protein hydrolysates prepared through enzymatic protein hydrolysis may have better absorption characteristics of proteins. Previous reports have shown that enzymatic protein hydrolysates acted as a suitable source of protein for human nutrition as the gastrointestinal tract absorbed the peptide more effectively when compared with free amino acids (Morris *et al*., 2007). Peptides present in protein hydrolysate of algae possessed several important bioactive activities included antioxidant (Harrysson *et al*., 2018), antibacterial, anti-inflammatory and antihypertension (Lee *et al*., 2015) . Previous studies reported that plants such as seaweed, wheat, mushrooms and spinach and bitter melon seeds used to obtain ACE inhibitory peptides through hydrolysis. Both in vivo and in vitro assays allow ACE inhibitory peptides derived from plants to demonstrate antihypertensive activity (Gupta *et al*.,2018).

2.5.2 Factors affecting degree of hydrolysis and bioactivity of protein

The main enzymatic hydrolysis parameters that influenced the hydrolysis of protein depends on the type protease used,hydrolysis time , temperature and pH (Benitez *et al*.,2008). These parameters will affect not only the degree of hydrolysis, but also the bioactivity and functional properties of protein (Admassu *et al*., 2015) and molecular weight distribution of the peptides (McCarthy *et al*., 2013). It is well known that the factors cooperatively influence the enzyme activity in protein hydrolysis, thereby making the process more controllable (Jamil *et al*, 2016)

2.5.2.1 Types of proteases

Bioactive peptides from seaweed can be obtained basically by protein hydrolysis using digestive proteases, proteases from plant or microbial proteases during the fermentation process (Samarakoon and Jeon, 2012). In enzymatic hydrolysis process, encrypted peptides are often discharged to play their specific role. Protease has a sensitive structure containing an active site that catalyzes specific substrates and performs highly specific reactions (Hasson *et al*., 2002). These proteolytic hydrolyzing enzymes can work either separately or in a serial combination of them for the production of bioactive peptides, which range from 2 to 20 amino acid compositions.

A large variety of commercial enzymes mainly endo-peptidase and exo-peptidases, also called endo-proteases and exo-proteases, are available for the production of protein hydrolysates. Endo-peptidases hydrolyze amino acids of the interior of the polypeptide chain, where as exo-peptidases hydrolyze from either the carboxyl end (C terminal) or the amino end (N terminal) of the protein. Although most commercial enzymes are endo-peptidases, the mixtures of endo-peptidases and exo-peptidases also can act as an enzyme (Hamada, 2000). A wide variety of proteolytic enzymes are available from fermentations, animal and plant commercially. The most commonly used enzymes for protein hydrolysates from plant sources are papain and bromelain, animal sources are pancreatin, trypsin and pepsin, and microbial fermentation sources are Alcalase. Protein hydrolysis can be accomplished in two ways include a single enzymatic step using an enzyme and a sequential enzyme hydrolysis using multiple enzymes. The choice of enzyme depends on the protein source and end user requirements (Pasupuleti and Demain, 2010).

2.5.2.2 pH

There are different parameters in protein hydrolysis and this makes it become a complex system to produce protein hydrolysates. The parameters include a mass of the hydrolysis mixture, E/S, pH, temperature and conditions needed to inactivate the protein hydrolysis process (Navarrete and Garcia, 2002). pH is an extremely crucial and sensitive parameter not only in biological, chemical and medical research areas but also in laboratory and industrial applications, especially the protein hydrolysis process. The enzyme is a type of protein that sensitive to pH change from the surroundings as the three-dimensional structure and amino acid functional groups in protein needs an optimum pH for more adequate to bind and catalyst the substrate (Bisswanger, 2014). The optimum pH for protease is a narrow range of pH and is usually determined through the bell-shaped curve in the graph of enzyme activity as a function of pH (Talley and Alexov, 2010). The most favorable physiological pH value for the most enzyme is 7, which is the neutral value. However, there are several exceptions. For example, Alcalase is an alkaline protease, so the pH range of Alcalase is alkaline within the range from 7 to 9 (Ma *et al*., 2015; Awuor *et a*l., 2017). Pepsin is an acid protease with an optimum pH from 2 to 3 (Jung *et al.*, 2014). The compound that resists the addition of acids and alkalis is known as a buffer. A buffer solution is used to adjust and control the pH value in proteolytic hydrolysis to allow the maximum activity of proteases for the production of protein hydrolysate (Okamoto *et al.*, 2017). As extreme pH, away from the optimum pH of a particular protease, this will result in complete loss of enzyme activity and denature.

2.5.2.3 Temperature

Over a century ago, the rate of enzyme activities and temperature applied to the enzymatic hydrolysis process are discovered. The degree of protein hydrolysis increase when the hydrolysis temperature increase as the higher the temperature, the higher the rate of reaction of an enzyme A temperature rise of 10ºC, doubles the reaction rate (Ovissipour *et al.*, 2009). When the temperature is low, the enzyme is inactive and lead to no hydrolysis process occurring. In contrast, the enzyme deactivates if the temperature is too high because of the intermolecular attraction between polar groups such as hydrogen bonding, dipole-dipole attractions and ionic interactions and hydrophobic forces between the non-polar groups of the enzyme break by the high thermal energy. The confirmation of the active site of the enzyme alters and most of the enzyme is thermal deactivation or denaturation at high temperature (Daniel and Danson, 2013; Robinson, 2015). The protein hydrolysis process is usually terminated by inactivating the protease through heat treatment. The adjustment of temperature avoids the production of unwanted subsequent peptides or protein hydrolysate and the development of harsh conditions (Jo et al., 2017). Different proteases have different optimum temperatures and germination temperatures.

2.5.2.3 Hydrolysis Time

The prolonged hydrolysis time or incubation time is directly proportional to the degree of hydrolysis (DH), when other parameters such as E/S, pH and temperature are constant. The amount of bioactive peptide becomes higher ,when the hydrolysis time given to the enzyme increase and further increase the functional properties such as ACE inhibitory activities of food product (Mohtar *et al*., 2014). Prior studious emphasize that the concentration of protease has a significant effect on reducing hydrolysis time. When the protease concentration is high, the protease will not become the limiting factor. Protease is free to bind with the substrate and increase the rate of protein hydrolysate production in a shorter hydrolysis time (Taylor *et al*., 2005). Throughout the protein hydrolysis process, the degree of hydrolysis (DH) increases proportionally with time. The rate of protein hydrolysis decreases slowly at the end of the process because the peptide bonds within the protein are no longer available for hydrolysis. The number of peptides and the native protein becomes constant as the same ratio of enzyme and substrate (Bao *et al.*, 2017). Table 2.7 shows the commercial enzyme and various optimum parameters (Amiza *et al*., 2017).

Table 2.7: Commercial enzyme and various optimum parameter (Amiza *et al*., 2017)

Enzyme	pH	Temperature (°C)	Hydrolysis time (hour)
Alcalase	8.5	55	2, 4
Protamex	6.5	50	2, 4
Neutrase	7.0	55	2, 4
Papain	6.0	60	2, 4

2.6 Bioinformatics approach

In order to bypass some challenges of the classical approach, computer-based (often known as “in silico”) simulation has been recently applied towards the invention of bioactive peptides encrypted in food proteins (Holton et al., 2013).With the advantage of

simultaneously evaluating multiple food proteins and proteolytic enzymes, bioinformatics is well-positioned to make a transformative impact in bioactive peptide research. The in silico approach involves the use of information accrued in databases, such as BIOPEP (Dziuba *et al*.,1999) to determine the occurrence frequency of cryptic bioactive peptides in the primary structure of food proteins. The protein sequences can be obtained from databases, notably the universal protein knowledgebase (UniProtKB)(Udenigwe, 2014). Other researchers also noted that bioinformatics tools can be used to select the critical process parameters for the production of specific peptides from proteins as well as to identify novel peptides with the aim of synthesizing and determining their bioactivity. This computer-based (or "in silico") approach utilizes information databases. BIOPEP-UWM is a specific database for bioactive peptides, which enables to reach specific peptide sequences with their potential activity. Several other databases can be used to obtain protein sequences for analyzing their potential bioactive peptides; such as UniProtKB, SwissProt, TrEMBL and NCBI (National Center for Biotechnology Information) (Kartal *et al*., 2020). Many researchers start to integrate in silico approach with experimental studies due to its time-saving and economical benefits and they have performed experimental studies which have supporting results within silico evaluations. Nowadays, bioactive peptides have gained importance as their potential impact on human health became clear and the integration of in silico approach in experimental studies to predict the possible positive health effects of bioactive peptides become one of the primary interests of researchers in worldwide (Kartal *et al*., 2020).

2.7 Bioactive peptide

Food-derived bioactive peptides commonly contain 2 to 20 amino acids and they are inactive when encrypted in their native protein structure. They need to be released by protein degradation. Peptides present in protein hydrolysate of algae possessed several important bioactive activities included antioxidant (Harrysson *et al*., 2018), antibacterial (Shannon and Abu-Ghannam, 2016), anti-inflammatory (Lee *et al*., 2015) and antihypertension (Suetsuna *et al*., 2004).

2.7.1 DPP IV inhibitor

Seaweed-derived peptides have been shown to be efficient in inhibiting the enzymes dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) and platelet-activating factor acetylhydrolase (PAF-AH; EC 3.1.1.47)(Lafarga *et al*., 2020). Dipeptidyl peptidase-IV (DPP- IV) inhibitors unique approach for the management of diabetes has been considered to be safe, as DPP-IV inhibitors reduce blood glucose levels by monitoring hyperglycemia including positive effects on weight because it remains neutral, improve glycated hemoprotein levels and don't induce symptom.(Singh *et al*., 2017).Seaweed-derived DPP-IV restrictive peptides known to this point enclose the peptides ILAP, LLAP, and MAGVDHI, recently rumored by Harnedy, Georgia Okeeffe and FitzGerald**.**The peptides ILAP, LLAP, and MAGVDHI were generated from an aqueous protein extract of *Palmaria palmata* using Corolase PP and showed DPP- IV EC50 values of 43.4, 53.7, and 159.4 μM, respectively. In addition, Fitzgerald, Gallagher, O'Connor, Prieto, Mora-Soler, Grealy and Hayes identified the PAF-AH inhibitory tetra-peptide NIGK, which was generated from *Palmaria palmata* using papain and showed a PAF-AH EC50 value of 2.3 mM (Lafarga *et al.*, 2020).

2.7.2 ACE inhibitory peptide

ACE(Angiotensin-I-Converting Enzyme) is a crucial enzyme in blood pressure regulation and leads to hypertension. Hence, the inhibition of ACE using ACE inhibitor is important to treat high blood pressure (Coppey *et al*., 2006). ACE inhibitors not only inactivate the formation of angiotensin II from angiotensin I but also increase bradykinin

bioavailability by reducing its degradation into inactive fragments (Gamboa *et al*., 2011). Thus, ACE inhibitors are always the first choice for patients in treating hypertension.

Natural food-based ACE inhibitors are safer than synthetic ACE inhibitors drugs such as captopril, enalapril and lisinopril because a study has reported that synthetic drugs may contain carcinogenic contaminants N-nitrosodimethylamine (NDMA) and other side effects (Packard *et al*.,2002). The previous report has shown that 20% of hypertension patients stop the ACE inhibitor treatments due to the occurrence of side effects, especially chronic cough (Morimoto *et al*., 2004). Therefore, the non-toxic, safer and economically friendly natural ACE inhibitors from food-based materials are more preferable. Researchers are more focused on the development of natural ACE inhibitors that are isolated from a variety of natural bio-resources and functional foods (Kumar *et al*., 2010). Previous studies reported that plants such as seaweed , wheat mushrooms (Jang *et al*., 2011), spinach (Yang *et al*., 2003) and bitter melon seeds used to obtain ACE inhibitory peptides through hydrolysis. Both in vivo and in vitro assays allow ACE inhibitory peptides derived from plants to demonstrate antihypertensive activity (Gupta *et al.*, 2018).

2.7.3 Antioxidative peptide

The oxidation of lipids and proteins of food products during processing or storage by reactive oxygen species (ROS), such as superoxide anion radical (O_2^{-}•), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), and Peroxyl radical (•OOR) ,is the major reason for food deterioration that would reduce consumer acceptability of food due to undesirable changes of quality and the possible production of toxic compounds. (Li *et al*., 2010). Consuming these potentially toxic products may trigger various human chronic diseases, including cancer, arteriosclerosis, aging diabetes mellitus, inflammation, coronary heart

diseases, and neurological disorders, such as Alzheimer's disease (Kitts *et al*., 2003). Therefore, to prevent food products from such deteriorations and protect consumers against serious diseases, one key strategy is inhibition of lipid peroxidation occurring in the living body and food products by using antioxidant substances or preservatives Antioxidants or preservatives are chemical components in biological materials, relatively found in low concentrations that prolong the shelf life of food by delaying or inhibiting the oxidation of a substrate in the food (Balboa *et al*.,2013). Bioactive peptides are the most apperaring antioxidative substances in food. Synthetic substances, for example, butylated hydroxyanisole(BHA), butylated hydroxytoluene (BHT), propyl gallate, TBHQ (tert-butyl hydroquinone) have better antioxidant activity and retarding effects of oxidation than those of the natural antioxidants. However, the use of these chemical antioxidants needs strict control due to their potential health risks and toxicity (Admassu *et al*., 2018). To date, no study has been reported on the evaluation of bioactive peptides from *Gracilaria changii* protein by using silico tools.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Study area

This study was done at Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka.

3.2 Material

3.2.1 Raw materials

One kilograms of dried seaweed (*Gracilaria changii*) was purchased from Malaysia. The seaweed was stored in chiller at 4^{o}C until further use.

3.3 Methods

3.3.1 Overview of study

Firstly, the seaweed was cleaned with water three times to remove all the contaminants, cut into small pieces and lyophilized, prior to grinding into powder form. Secondly, seaweed protein was extracted using cellulase and ammonium sulphate treatment, prior to freeze drying and MALDI- TOF mass spectrometry analysis.Then,bioactive peptide was predicted from target protein phycocyanin via “In silico “ approach..

3.3.2 Preparation of raw material

According to Chan and Matanjun (2017), the dried seaweed was washed with tap water three times to remove all the unwanted impurities, adhering sand particles, epiphytes and other contaminants. Secondly, the seaweed was cut into small pieces of around 1 cm long. Thirdly, the excess water was removed by placing the wet seaweed in a siever and

then the seaweed was wrapped in aluminium foil, followed by storage in a freezer at -80^{o}C. Then, the seaweed was freeze-dried using a cabinet freeze dryer. Next, dry blender was used to grind the lyophilized seaweed into powder form. Lastly, the seaweed was stored in chiller for further use.

3.3.3 Protein extraction of seaweed

Protein extraction method from seaweed was employed as described by Galland-Irmouli *et al*. (1999), with slight modification. The method used a combination of treatment using cellulase enzyme, sonication and ammonium sulphate extraction. Ten grams of freeze-dried and ground seaweed powder was suspended in 250 mL acetate buffer with 1 g cellulase for 2 h by using water bath(Shaker bath 903, Protech, UK). Next, the mixture was suspended in 1 L of ultrapure water independently, followed by sonication for 1 h. Then, the seaweed solution was stirred overnight on a magnetic stirrer plate at 4°C. The seaweed solution was centrifuged at 10,000×g for 30 min and the supernatant was decanted. The supernatant was brought to 60% (w/v) ammonium sulphate saturation. The mixture was stirred at 4°C for 1 h, follow by centrifuged at 10,000×g for 30 min to precipitate the protein fraction. The precipitates were dialyzed using 3.5-kDa MWCO dialysis tubing (Fischer Scientific, USA) against ultrapure water at 4°C overnight. Finally, the precipitate was freeze-dried and stored at - 80°C until further use.

3.3.4 Protein identification from *Graciliria changii* protein by MALDI-TOF/MS

Extracted protein wereanalyzed by using matrix- assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS). MALDI as a principle for analysis of

large bio- molecules was introduced by Karas and Hillenkamp. Briefly, in MALDI-MS, the sample is embedded in the crystalline structure of small organic compounds (matrix) and deposited on a conductive sample support. The cocrystals are irradiated with a nanosecond laser beam, for example, an ultraviolet (UV) laser with a wavelength of 266 or 337 nm. The energies introduced are in the range of 1 × 107–5 × 107 W/cm2. The laser energy causes structural decomposition of the irradiated crystal and generates a particle cloud (the plume) from which ions are extracted by an electric field. Following acceleration through the electric field, the ions drift through a field-free path and finally reach the detector (e.g., asecondary electron multiplier or channel plate). Ion masses typically calculated by measuring their TOF, which is longer for larger molecules than for smaller ones.(Jurinke *et al.*, 2004)

3.3.5 In silico analysis

The target *Graciliria changii* protein alpha phycocyanin subunit was identified by MALDI-TOF/MS data analysis.Itwas further investigated using NCBI(Nationalcenter for Biotechnology information (https://www.ncbi.nlm.nih.gov) and BIOPEP-UWM

database(https://biochemia.uwm.edu.pl/biopep-uwm) to predict the potential biological activities.Firstly, protein sequence of alpha phycocyanin subunit obtained from NCBI database.Then,"Bioactive peptides" was chosen from the "database" options of BIOPEP-UWM.The identified target protein from analyzed using the "profiles of potential biological activity" tool, and the name of peptide, activity, number of peptide, sequence and location of bioactive peptides in protein sequences were acquired. The occurrence of frequency (A) of bioactive peptides was calculated as A = a/N, where a = number of bioactive peptides and N = total number of amino acid (AA) residues in the protein chain (Panjaitan et al., 2018).

CHAPTER 4: RESULTS

4.1 Extracted Protein identified by MALDI-TOF mass spectrometry

MALDI-TOF analysis revealed that the extracted *Gracilaria changi* protein include C-phycocyanin beta chain, C-phycocyanin alpha chain, Malate dehydrogenase, Ribolose-bisphosphate -carboxylase and Allophycocyanin alpha chain. These are listed in the Table with their NCBI ID, molecular mass and amino acid number.

Table 4.1:*Gracilaria changii* protein predicted by MALDI-TOF mass spectrometry

SL.	Protein	NCBI ID	Molecular Mass(Da)	AA No.
1	C-phycocyanin beta chain	45777	18,201	172
2	C-phycocyanin beta chain	45778	18,185	172
3	C-phycocyanin alpha chain	45780	17,464	162
4	Malate dehydrogenase	46467	35,037	326
5	Ribulosebisphosphate carboxylase large chain	45785	51,379	467
6	**A**llophycocyanin alpha chain	46453	17,532	161

4.2 In silico analysis

The biopep analyses showed that there were 175 bioactive peptides with several types of biologically functional peptides in phycocyanin alpha subunit.The numbers of peptides released from phycocyanin alpha subunit are presented in Table 4.2

Table 4.2: Evaluation of potential bioactive peptides of *Gracilaria changii* protein by BIOPEP-UWM database (accessed on November,2021).

Name of peptide	Peptide Sequence
DPP IV inhibitor	94 GP (2), MP (1), KA (1), VV (1), TP (1), SP (1), FP (1), GA (2), IA (2), RA (1), FL (1), AL (2), SL (3),GL (1), AA (3), WY (1), YT (1), AD (1), AG (1), AS (4), AT (1), EI (1), EY (1), GE (1),GY (1), HS (1), IN (3), KF (1), KT(1), LI (2), LT (1), LV (1), MK (1), NA (2), NE (1), NG (2), NR (1), NT (1), PF (1), PG (1), PI (1), PM (1), PS (1), PT (1), QA (1), QG (1), QS (2), QY (1), RL (1), RM (1), SF (1), SH (1), SI (1), SV (1), SVV (1), TG (2), TK (1), TQ (1),TS (1), TT (2), TY (3), VG (1),VY (1),YA (2),YI (2),YL (3),YQ (1),YY(1), GPM (1).
Ace Inhibitors	68 RY (1), RF (1),VY (1), FP (1), LSP (1),YL (3), DIGYY (1), GP (2), RA (1), IA (2),YA (2),AA (3), VG (1), IG (2), GA (2), GL (2), AG (1), GR (2), GQ (1) ,GK (1), GE (1), QG (1),SG (1),TG (2), EA (4), NG (2),PG (1), SF (1), KF (1), AR (2), KA (1), EY (1), EI (1), IE(1), TE (1), LQ (2), PT (1), TQ (1), AI (3), RYQ (1), FTTQ (1), ASL (1), LEE (1),SVY (1), GPM(1), DY(1),TP (1),TGP (1), LR (1), LDY (1), SVYT (1),YY (1),YLR (1), LRM (1).
DPP III inhibitors	13 YY(1),LR(1),YL(3),GE(1), RF(1),FL(1),PF(1),VY(1),YI(2),KA(1)
Antioxidative	13 GYY (1), EL (2), TY (3), VY (1), LDY (1), RYQ (1), GAA (1), CLV (1), SVYT (1), RY (1)
Renin inhibitors	8 FT (1), AR (1), KF(1), NR (1),SF (1),YA (2), SVYT (1)
PEP inhibitors	4 PGP (1), PG (1), GP (2)

®PEP= Prolyl endopeptidase

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The sequence of peptide identied from *Gracilaria changii* protein and their chracteristics, i.e.activity, EC_{50}(Inhibition of half maximum actviity), location and number of occurrence are presented in Table 4.3

Table 4.3: Sequence of peptide identified from *Gracilaria changii* protein and their chracteristics

Sequence of peptide	Activity	EC_{50} (µM)	Location	No.	Reference
GP	DPP IV inhibitor	9690.00	[71-72],[105-106]	2	Gallego *et al*., 2014
	Antithrombotic	0.00			Ashmarin *et al.*,1998
	Antiamnestic	0.00			Ashmarin *et al*.,1998
	ACE inhibitor	252.63			Byun *et al*., 2002
MP	DPP IV inhibitor	870.00	[69-70]	1	Hatanaka *et al*.,2012
KA	DPP IV inhibitor	6270.00	[81-82]	1	Gallego *et al*., 2014
	ACE inhibitor	31.50			Sentandreu *et al*.,2007
	DPP III inhibitor	0.00			Dhanda *et al*., 2009
VV	DPP IV inhibitor	0.00	[100-101]	1	Bella *et al*., 1982
TP	DPP IV inhibitor	2370.00	[3-4]	1	Hatanaka *et al.*,2012
	ACE inhibitor	288.40			Wu *et al*., 2008
SP	DPP IV inhibitor	5980.00	[125-126]	1	Hatanaka *et al*., 2012
FP	ACE inhibitor	315.00	[63-64]	1	Abubakar *et al.*, 2010
	DPP IV inhibitor	363.00			Hatanaka *et al.*, 2012
GA	ACE inhibitor	2000.00	[54-55], [102-103]	2	Cheung *et al*.,1980
	DPP IV inhibitor	0.00			Hikida *et al*., 2013
IA	ACE inhibitor	153.00	[9-10],[112-113]	2	Hikida *et al*., 2013
	DPP IV inhibitor	0.00			Hikida *et al.*, 2013

RA	Activating ubiquitin-mediated proteolysis	0.00 460.00	[33-34]	2	Turner *et al.*,2000 Cushman D. W., 1981
	ACE inhibitor	0.00			Hikida *et al*., 2013
	DPP IV inhibitor	0.00			Lan *et al.*, 2015
FL	DPP IV inhibitor	399.58	[18-19]	1	Nongonierma A. B., 2013
	DPP III inhibitor	0.00			Dhanda S., 2008
AL	DPP IV inhibitor	882.13	[132-133],[160-161]	2	Nongonierma *et al*.,2013
SL	DPP IV inhibitor Regulating	2517.08 0.00	[37-38],[43-44],[141-142]	3	Luzarowski *et al.*, 2021
GL	ACE inhibitor DPP IV inhibitor	2500.00 2615.00	[114-115]	1	Cheung *et al*., 1980 Nongonierma *et al.*, 2013
AA	ACE inhibitor DPP IV inhibitor	620.00 9400.00	[40-41],[55-56],[146-147]	3	Cushman D.W., 1981 Gallego *et al*., 2014
WY	Antioxidative DPP IV inhibitor	0.00 281.00	[128-129]	1	Hernandez *et al*., 2007 Nongonierma *et al.*,2007
YT	DPP IV inhibitor	0.00	[60-61]	1	Nongonierma *et al.*,2014
AD	DPP IV inhibitor Alpha-glucosidase inhibitor	0.00 25660.00	[12-13]	1	Lan *et al.*, 2015 Mora *et al.*, 2020
AG	ACE inhibitor DPP IV inhibitor	2500.00 0.00	[113-114]	1	Cheung *et al*., 1980 Lan *et al*., 2015
AS	DPP IV inhibitor	0.00	[10-11],[34-35],[36-37],[75-76]	4	Lan *et al*., 2015
AT	DPP IV inhibitor	0.00	[103-104]	1	Lan *et al*., 2015

EI	ACE inhibitor	0.00	[117-118]	1	van Platerink *et al*,2008
	DPP IV inhibitor	0.00			Lan *et al*., 2015
EY	ACE inhibitor	2.68	[109-110]	1	Wu *et al*., 2008
	DPP IV inhibitor	0.00			Lan *et al*.; 2015
GE	ACE inhibitor	5400.00	[22-23]	1	Cheung et al., 1980
	DPP IV inhibitor	0.00			Lan *et al*., 2015
	DPP III inhibitor	0.00			Dhanda *et al*., 2008
GY	ACE inhibitor	210.00	[89-90]	1	Cheung *et al*., 1980
	DPP IV inhibitor	0.00			Lan et al., 2015
IN	DPP IV inhibitor	0.00	[27-28],[118-119],[158-159]	3	Lan *et al*., 2015
KF	ACE inhibitor	28.30	[62-63]	1	Meisel *et al*., 2006
	Renin inhibitor	0.00			Li H *et al*., 2010
	CaMPDE inhibitor	0.00			Li H *et al*., 2010
	DPP IV inhibitor	0.00			Lan *et al*., 2010
KT	DPP IV inhibitor	0.00	[2-3]	1	Lan *et al*., 2015
LI	Stimulating	0.00	[51-52],[111-112]	2	Morifuji *et al.*, 2009
	DPP IV inhibitor	0.00			Lan *et al*., 2015
LT	DPP IV inhibitor	0.00	[44-45]	1	Lan *et al*., 2015
LV	Stimulating	0.00	[99-100]	1	Morifuji *et al*., 2009
	DPP IVinhibitor	0.00			Lan *et al*., 2015
HS	DPP IV inhibitor	0.00	[140-141]	1	Lan *et al*., 2015
MK	DPP IV inhibitor	0.00	[1-2]	1	Lan *et al*., 2015
NA	DPP IV inhibitor	0.00	[47-48],[159-160]	2	Lan *et al*., 2015
NE	DPP IV inhibitor	0.00	[148-149]	1	Lan *et al* ., 2015

NG	ACE inhibitor	12000.00	[21-22],[28-29]	2	Cushman D. W., 2009
	DPP IV inhibitor	0.00			Lan *et al*., 2015
NR	DPP IV inhibitor	0.00	[119-120]	1	Lan *et al*., 2015
	Renin inhibitor	0.00			Udenigwe *et al.*, 2012
NT	DPP IV inhibitor	0.00	[151-152]	1	Lan *et al*., 2015
PF	DPP IV inhibitor	0.00	[64-65]	1	Lan *et al*., 2015
	DPP III inhibitor				Dhanda *et al*., 2008
PG	Antithrombotic	0.00	[70-71]	1	Ashmarin *et al.*,1998
	Antiamnestic	0.00			Ashmarin *et al*., 1998
	ACE inhibitor	17000.00			Cheung *et al*., 1980
	DPP IV inhibitor	0.00			Lan *et al*., 2015
PM	DPP IV inhibitor	0.00	[106-107]	1	Lan *et al*.,2015
PI	DPP IV inhibitor	0.00	[4-5]	1	Lan *et al*.,2015
PS	DPP IV inhibitor	0.00	[126-127]	1	Lan *et al*., 2015
PT	ACE inhibitor	0.00	[72-73]	1	VanPlaterink *et a*l., 2008
	DPP IV inhibitor	0.00			Lan *et al*., 2015
QA	DPP IVinhibitor	0.00	[145-146]	1	Lan *et al*., 2015
QG	ACE inhibitor	7400.00	[15-16]	1	Cushman D. W., 1980
	DPP IV inhibitor	0.00			Lan *et al*., 2015
QS	DPP IV inhibitor	0.00	[25-26],[57-58]	2	Lan *et al*., 2015
QY	DPP IV	0.00	[50-51]	1	Lan *et al*., 2015

	inhibitor				
RL	ACE inhibitor	2439.00	[50-51}	1	Lan *et al*., 2015
	DPP IV inhibitor	0.00			Lan *et al.*, 2015
RM	DPP IV inhibitor	0.00	[93-94]	1	Lan *et al*., 2015
SF	ACE inhibitor	130.20	[121-122]	1	Suetsuna *et al.*, 1998
	DPP IV inhibitor	0.00			Lan *et al*., 2015
	Renin inhibitor	0.00			Udenigwe *et al* ., 2012
SH	DPP IV inhibitor	0.00	[139-140]	1	Lan *et al*., 2015
SI	DPP IV inhibitor	0.00	[26-27]	1	Lan *et al*., 2015
SV	DPP IV inhibitor	0.00	[58-59]	1	Lan *et al*., 2015
SW	DPP IV inhibitor	0.00	[127-128]	1	Lan *et al.*, 2015
TG	ACE inhibitor	9900.00	[53-54],[104-105]	2	Cheung *et al*., 1980
	DPP IV inhibitor	0.00			Lan *et al*., 2015
TK	DPP IV inhibitor	0.00	[61-62]	1	Lan *et al*., 2015
TQ	ACE inhibitor	0.00		1	van Platerink C. J., 2008
	DPP inhibitor	0.00	[67-68]		Lan *et al*., 2015
TS	DPP IV inhibitor	0.00	[45-46]	1	Lan *et al.*, 2015
TT	DPP IV inhibitor	0.00	[66-67],[95-96]	2	Lan *et al*., 2015
TY	Antioxdative	0.00	[73-74],[96-97],[152-153]	3	Cheng *et al*., 2010
	DPP IV inhibitor	0.00			Lan *et al.*, 2015
VG	ACE inhibitor	0.00	[101-102]	1	Cheung *et al*., 1980
	DPP IV inhibitor	0.00			Lan *et al*., 2015
VY	ACE inhibitor	7.10	[59-60]	1	Saito *et al.*, 1994
	Antioxidative	0.00			Cheng *et al*., 2010
	DPP IV	0.00			Lan *et al*., 2015

	inhibitor				
	DPP III inhibitor	0.00			Dhanda *et al*., 2008
YA	ACE inhibitor	0.00	[74-75],	2	Cushman D. W., 1980
	DPP IV inhibitor	0.00	[156-157]		Lan *et al*., 2015
	Renin inhibitor	0.00			Udenigwe *et al.*, 2012;
YI	DPP IV inhibitor	0.00	[129-130],[135-136]	2	Lan *et al*., 2015
	DPP III inhibitor	0.00			Lee,*et al*., 1982
YL	ACE inhibitor	122.00	[91-92],[110-111],[153-154]	3	Mullally *et al*., 1996
	Neuropeptide	0.00			Kanegawa *et al.* , 2010
	DPP IV inhibitor	0.00			Lan *et al*., 2015
	DPP III inhibitor	0.00			Lee *et al*., 2015
YQ	DPP IV inhibitor	0.00	[31-32]	1	Lan *et al.*, 2015
YY	DPP IV inhibitor	0.00	[90-91]	1	Lan *et al*., 2015
	DPP III inhibitor	0.00			Lee *et al*., 1982
	ACE inhibitor	180.00			Lafarga *et al*., 2016
GPM	ACE inhibitor	16.98	[105-107]	1	Wu J *et al*., 2006
	DPP IV inhibitor	417.90			Jin *et al*., 2015
RY	ACE inhibitor	10.50	[30-31]	1	Cheung *et al*., 1980
	DPP IV inhibitor	0.00			Lan *et al*., 2015
RF	ACE inhibitor	93.00	[17-18]	1	Saito *et al.*, 1994
	DPP III inhibitor	0.00			Dhanda *et al*., 2008
GQ	Neuropeptide	0.00	[30-31]	1	Parish *et al*., 1983
	ACE inhibitor	7000.00			Cheung *et al*., 1980
GK	ACE inhibitor	5400.00	[22-23]	1	Cheung *et al*., 1980
SG	ACE inhibitor	8500.00	[143-144]	1	Cheung *et al*., 1980

EA	ACE inhibitor	10000	[7-8],[39-40],[131-132],[149-150]	4	Cheung *et al*., 1980
	Alpha-glucosidase inhibitor	17000			Mora L., 2020
AR	ACE inhibitor	95.50	[130-131]	1	Sentandreu *et al* ., 2007
KA	DPP IV inhibitor	6270.00	[81-82]	1	Gallego *et al*., 2014
	ACE inhibitor	31.50			Sentandreu *et al*., 2007
	DPP III inhibitor	0.00			Dhanda *et al*., 2008
IE	ACE inhibitor	0.00	130-131]	1	van Platerink *et al*., 2008
TE	ACE inhibitor	0.00	[6-7]	1	van Platerink *et al*., 2008
	DPP IV inhibitor	0.00			Lan *et al*., 2015
LQ	ACE inhibitor	0.00	[24-25],[133-134]	2	van Platerink *et al*., 2008
AI	ACE inhibitor	3.41	[8-9],[78-79],[157-158]	3	Nakahara T., 2010
DY	Regulating	0.00	[155-156]	1	Ziganshin *et al.*, 1994
	ACE inhibitor	100.00			Wu *et al*., 2006
LR	Renin inhibitor	0.00	[92-93]	1	Udenigwe *et al*., 2012
	ACE inhibitor	158.00			Liu *et al*., 2014
	DPP III inhibitor	0.00			Lee *et al*., 2012
ASL	ACE inhibitor	102.15	[36-38]	1	Wu *et al*., 2015
RYQ	ACE inhibitor	0.00	[30-32]	1	De Gobba *et al*., 2014
	Antioxidative	0.00			Liu *et al*., 2015
FTTQ	ACE inhibitor	0.00	[65-68]	1	Mojica *et al*., 2015
LEE	ACE inhibitor	100.00	[115-117]	1	Wu J *et al*., 2006
SVY	ACE inhibitor	8.13	[58-60]	1	Wu J *et al*., 2006
TGP	ACE inhibitor	79.10		1	O'Keeffe M. *et al.*, 2017
LDY	Antioxidative	0.00	[154-156]	1	Liu *et al*., 2015
SVYT	ACE inhibitor	63.00	[58-61]	1	Girgih *et al*., 2014

	Antioxidative	0.00			Girgih *et al*., 2014
	Renin inhibitor	0.00			Girgih *et al*., 2014
YLR	ACE inhibitor	5.80	[91-93]	1	Kumagai *et al*., 2021
LRM	ACE inhibitor	0.15	[92-94]	1	Kumagai *et al*., 2021
GYY	Opioid	1000.00	[89-91]	1	Fukudome *et al*.,1992
	Antioxidative	0.00			Yokomizo *et al* ., 2002
FT	Renin inhibitor	0.00	[65-66]	1	Udenigwe *et al*., 2012

CHAPTER 5: DISSCUSSION

In the present study, an attempt was to evaluate potential bioactive peptides from alpha phycocyanin (protein) of *Gracilaria changii*.

5.1 Protein identification from *Gracilaria changii* protein by MALDI-TOF/MS

Table 4.1 listed the identified protein with their accession number, molecular weight and amino acid number. The selected protein phycocyanin -alpha contains molecular weight 17,464(Da) and 162 amino acids.

5.2 In silico analysis

Table 4.2 indicates the peptide name and their sequence. Fifteen biological activitieswere identified from the biopep analysis. Six of them, i.e. DPP-IV inhibitor, Ace inhibitor,DPP III inhibitior, antioxidative , renin inhibitor and Prolyl endopeptidase (PEP) inhibitor. The result also showed that DPP-IV and ACE inhibitory peptide were high in number respectively 94 and 64 exist in phcocyanin. Table 4.3 represents the sequence of peptide , activity, EC50 value ,location in parents protein and number of occurrence. Almost all DPP-IV inhibitory peptides exhibited most potent activities containing EC50 value 0.00µM except 8 DPP-IV inhibitory peptides, which possess higher EC50 value(EC50>300µM),those peptides were di-peptide GP,KA,NG,GL,SL,AL,FP and tri-peptide GPM.The ACE inhibitory peptides GA,AG,GE,NG,QG,RL,TG,GQ,GK,SG and EA contain higher EC50 value (EC50>2000µM), rest of all contain lower EC50 value.

5.2.1 DPP-IV inhibitory peptide

In the result DPP-IV is predominated bioactive peptide containing lower EC_{50} value. In the previous literature reported that Dipeptidyl peptidase IV (DPP-IV) is involved in incretin hormone processing and therefore plays a key role in glycemic regulation.(Nongonierma & FitzGerald, 2019).Many researchers also added that DPP-IV inhibitory activities had the potential for management of cardiovascular disease, oxidative stress, type 2 diabetes and nervous system disorders, respectively.(Fu et al., 2016).

5.2.2 ACE inhibitory peptide

The frequency results showed that more than one-third part was comprised of antihypertensive effective peptides (ACE inhibitory peptides). Hypertension is generally recognized as a threat factor for cardiovascular diseases such as coronary artery disease, myocardial infarction, and stroke. Since it is crucial in controlling blood pressure, a dipeptidyl carboxypeptidase (also known as angiotensin-converting enzyme (ACE), (E.C. 3.4.15.1.) is one of the major protection pathways for hypertension (Verdecchia *et al.* 2008). It plays an important function in the rennin-angiotensin system. It also inactivates the vasodilator effect of the bradykinin hormone by degradation (Agirbasli and Cavas, 2017).

5.2.3 Other bioactive peptides

The result revealed that antioxidative peptide, DPP-III inhibitory peptide and renin inhibitory peptide were lower in number. The peptide of those also contains an EC_{50} value of 0.00 µM. Previous researchers reported that food products become toxic by

reactive oxygen. Those toxic products cause various human chronic diseases, including cancer, arteriosclerosis, aging, diabetes mellitus, inflammation, coronary heart diseases, and neurological. Therefore, to prevent food products from such deteriorations and protect consumers against serious diseases, one key strategy is inhibition of lipid peroxidation occurring in the living body and food products by using antioxidant substances or preservatives (Li-Chan , 2015). Antioxidants or preservatives are chemical contains components in biological materials, relatively found in low concentrations that prolong the shelf life of food by delaying or inhibiting the oxidation of a substrate in the food. Bioactive peptides are the most commonly occurring antioxidant substances(Admassu *et al*., 2018).

CHAPTER 6 : CONCLUSIONS

In evaluation of *Gracilaria changii* protein, phycocyanin released bioactive peptides. These bioactive peptides with DPP-IV inhibitory, ACE inhibitory, antioxidant, renin inhibitor and neuropeptide inhibitory activities had the potential for management of type 2 diabetes, cardiovascular disease and oxidative stress. This research showed that *Graciliria changii* protein are suitable for further investigations to find various bioactive peptides. According to the findings, protein of *Gracilaria changii* could be evaluated in the development of functional food or pharmaceutical products due to their therapeutic effects on type- 2 diabetes and cardiovascular and neurodegenerative diseases. The result of in silico analyses indicates that in vitro studies are needed to obtain DPP-IV and ACE inhibitor, antioxidative and neuroprotective peptides from *Gracilaria changii* protein. Synthetic peptides and drug molecules pose multiple challenges, including instability in the gastrointestinal tract, high cost, adverse side effects, stringent regulatory compliances, and intense market competition. Hence, the increasing demand for natural bioactive peptides necessitates the use of natural resources like seaweed as well as the redesign and development of an effective bioactive peptides production strategy that is feasible for nutraceutical and pharmaceutical applications.

CHAPTER 7 : RECOMMENDATIONS FOR FURTHER STUDY

More studies should be carried out on the *Gracilaria changii* seaweed protein extract in order to generating pharmaceuticals and functional products or the value-added products from *G. changii* seaweed protein. The suggestions for further studies are as follow:

- Study on generation of potential bioactive peptides from *Gracilaria changii* protein, specially with DPP-IV and ACE inhibitory peptides should be carried out.
- Study on potential for development of bioactive peptides such as antioxidant, anti-diabetics, and antimicrobial properties from seaweed protein extract should be performed.
- Study on physiochemical properties of seaweed powder can be examined.

  Study on physicochemical properties of seaweed protein extract can be performed.
- Study on fractionation and purification of digestive enzymes from seaweed protein extract can be implemented.
- Investigation on the identification on the active constituents of the seaweed protein extract (amino acid sequence) and determination of mechanism of ACE inhibitory action should be explored.
- Study on developing pharmaceutical products to increase their market value and to provide enhancement to human health.

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