In silico analysis of Gracilaria changii proteins for potential bioactive peptides

K N Sharmin¹, M A Amiza¹*, F Ahmad¹, S A Razali² and F Hashim²

¹Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Kuala Nerus. Terengganu 21030, Malaysia
²Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, Kuala Nerus. Terengganu 21030, Malaysia

*Corresponding author: ama@umt.edu.my

Abstract. Gracilaria changii is a red seaweed species in Malaysia with high protein content (12.57% (dry basis)). Thus, G. changii proteins are potential precursors for producing bioactive peptides. To date, no study has been reported on the potential of G. changii proteins as potential precursors for bioactive peptides. In this study, fourteen G. changii proteins were selected as potential precursors of bioactive peptides using in silico approach. It was found that the most potential bioactivity was dipeptidyl peptidase-IV (DPP IV) inhibitory and angiotensin-I converting enzyme (ACE) inhibitory activities. Papain, ficin and stem bromelain were used for in-silico proteolysis. Stem bromelain was found to be more effective in terms of the release of fragments with a given activity. Furthermore, two triptides (ACF and YCL) were screened as novel and promising bioactive peptides. The characteristics of both peptides were also analyzed using PeptideRanker, PepCalc, Peptide Cutter, ToxinPred, AllerTop and AHTpin bioinformatic tools. The bioinformatic tools predicted that both peptides were non-toxic, non-allergen and highly potential. The present work suggests that G. changii can serve as a potential source of bioactive peptides and these findings can provide a basis for future in-vitro and in-vivo study of bioactive peptides from G. changii proteins.

Keywords: ACE inhibitor; bioactive peptides; DPP-IV inhibitor; Gracilaria changii; in silico

1. Introduction

China, Japan and Korea used seaweeds as human food for ages [1]. According to their pigmentation, seaweed can be classified into three different taxonomic groups such as Rhodophyceae, Phaeophyceae and Chlorophyceae reflects red, brown and green colour respectively. In 2014, 28.5 million ton of seaweeds were harvested for the human consumption [2]. Besides human consumption, seaweeds play vital role in aquatic ecosystem as well. Seaweed also contains polysaccharides, pigments, fatty acids, polyphenols, bio-active peptides and various metabolites with therapeutic values. It has been proven that seaweeds possess biological valuable active properties to be considered as nutraceuticals and functional food [3]. Among the diverse bioactivities in seaweeds are antihypertension, immunomodulatory, antiinflammatory, antioxidant, anticancer and antimicrobial activities [4,5].

Bioactive peptides usually consisted of 2-30 amino acid. Once ingested in our body, they may give positive health effect [6]. In recent year, there is a growing interest on bioactive peptides from marine resources to be developed as new drugs and foods [7]. In general, red seaweed contains higher protein than other seaweed groups [8]. Thus, red seaweed could be an excellent source of bioactive peptides [9]. Bioactive peptides are inactive within the sequence of parent protein and have the ability to exert physiological properties on human beings once released by proteolysis [10,11]. Seaweed bioactive peptides are usually released by specific proteases hydrolysis. In-vitro hydrolysis of seaweed protein to generate bioactive peptides has been reported for Pyropia columbina [12], Gracilariopsis lemaneiformis [13], Ulva intestinalis [14], Undaria pinnatifida and Porphyra yezoensis [15,16].
In current era, various in silico proteolysis tools such as BIOPEP-UWN are utilized to envisage probable peptides released by proteases with a particular cleavage site from identified protein sequences. In silico tools resulted in reduced required time to screen for the potential peptides and is essential to detect of novel and valuable precursors of bioactive peptides [17]. BIOPEP-UWN incorporates a database of sequence, whereby its application permitting the construction of outlines of the ability biological activity of protein fragments, calculations of quantitative signifiers as measures of the value of proteins as potential precursors of bioactive peptides, and predictions of bonds susceptible to hydrolysis by endopeptidases in a protein chain[18]. BIOPEP-UWN has been used for the prediction of numerous proteins having angiotensin-converting enzyme (ACE) inhibitory activity, dipeptidyl peptidase-IV inhibitors (DPP-IV) and other bioactivities [19]. In silico analyses have been reported to study various potential peptides released from food-derived proteins such as green algae Caulerpa [20], yak milk casein [21] bovine collagen [22], crude barley protein [23] and rice bran protein [24].

Gracilaria, a red seaweed is a commercially important seaweed species in Southeast Asian countries, mainly used for production of agar [25]. Besides, Gracilaria changii showed cholesterol lowering properties [26]. Protein content (12.57±1.31%DW) of G. changii is higher than other Gracilaria species [25,27–30]. Fifteen main proteins with 14 accession numbers have been isolated by two- dimensional gel electrophoresis (2-DE) from G. changii. To date, there is no report in-silico analysis of G. changii proteins as precursors for bioactive peptides. Thus, the purpose of this study was to determine probable bioactive peptides released from previously identified G. changii proteins [30] and to determine the appropriate proteases that contributed to higher release of a dominant bioactive peptides. Furthermore, the study also explored in the prediction of novel peptide with their potential characteristics using in-silico bioinformatics tools.

2. Materials and Methods

2.1. Protein sequences of red seaweed (G. changii)

Fourteen proteins as identified by Wong et al. [31] were selected and confirmed with the NCBI database (https://www.ncbi.nlm.nih.gov/). Protein sequences in FASTA format and general characteristics of the 14 proteins were collected through UniPort database (https://www.uniprot.org/).

2.2. Evaluation of G. changii protein as potential precursor of bioactive peptides through BIOPEP-UWM database

The probability of the selected protein sequences to release bioactive fragments was determined using the BIOPEP-UWM database, which contains 4325 known bioactive peptides with 56 biological activities (accessed on 3rd August, 2021). The number of potential bioactive peptides were calculated using ‘profiles of potential biological activity’ action menu in BIOPEP-UWM. All the predicted activity of each protein was analysed. The fragment with the specific activity was count manually. Most frequent activities were chosen to report.

The frequency of bioactive fragments occurrence (A) in G. changii protein sequence was calculated using the following equation:

\[ A = \frac{a}{N} \]

where a is the number of fragments with given activity in a protein sequence, and N is the number of amino acid residues of protein. The total frequency of bioactive fragments in each fourteen protein sequences (ΣA) was also calculated. The incidence of bioactive fragments in G. changii protein sequence for the ACE and DPP IV inhibitor was calculated separately. The potential biological activity refers the valuable effects (such as ACE inhibitor, antioxidative, DPP IV inhibitor etc.) of the protein. The number of probable bioactive for each sub classes bioactivity was counted manually from BIOPEP-UWM analysis for the two bioactivities (bioactivities where B values is available). The potential biological activity of protein (B) was calculated using the following equation:
\[
B = \frac{\sum_{i=1}^{k} a_i \cdot EC_{50i}}{N}
\]

(2)

where \(a_i\) is the number of repetitions of \(i\)-th bioactive fragment in protein sequence, \(^{*}EC_{50}\) is the concentration of \(i\)-th bioactive peptide corresponding to its half-maximal activity [\(\mu\)M] or half-maximal inhibition (IC\(_{50}\)) in case of peptides with inhibitory activity, \(k\) is the number of different fragments with given activity, and \(N\) is the number of amino acid residues. BIOPEP-UWM only gives \(B\) values for ACE inhibitor, DPP IV inhibitor, hypotensive, alpha-glucosidase inhibitor and opioid.

2.3 In silico proteolysis

In-\textit{silico} proteolysis was accomplished using BIOPEP-UWM’s ‘enzyme/s action’ tool. Papain, ficin, and stem bromelain were independently used to the dominant protein sequences to release peptides. Predicted degree of hydrolysis (DH \%) was measured by the database. To assess the efficiency of release of bioactive fragments, the frequency of the bioactive fragments by the selected enzymes (\(A_w\)) and the relative frequency of the released peptides with given activity by selected enzymes (\(W\)) were calculated according to this equation:

\[
W = \frac{A_E}{A}
\]

(3)

where \(d\) is the number of peptides with given activity released from the protein sequence by selected enzyme, and \(N\) is the number of amino acid residues in the protein.

\[
A_E = \frac{d}{N}
\]

(4)

2.4 Virtual screening and characterization of novel tri-peptide

The bioactive fragments predicted to be released from \textit{G. changii} protein with their known activity were counted manually. BIOPEP-UWM gives the fragments with the bioactivities available in the database. Most of the reported sequences in BIOPEP-UWN were di or tri peptide for the activity of DPP-IV inhibitory activity. The peptide length of five amino acids and lower has shown to be potential for the dipeptidyl peptidase IV (DPP-IV) inhibitors ([32,33]). Therefore, to further investigate for novel tri-peptide after proteolysis with the selected enzyme, the fragments with three amino acid was submitted to rank for the potential activity as previously described by Lafarga [32]. Peptide Ranker is available at http://distilldeep.ucd.ie/PeptideRanker/. It is a bioinformatic tool that predicts and ranks possibility of a peptide to be bioactive based on the a on an N-to-1 neural network algorithm [34]. One of the limitations of the tool that it does specify the activity that are most suitable. Furthermore, the literature review carried out the suitable activity of the peptides shown in the table 1.

Furthermore, in \textit{silico} analysis was performed to determine the potential characteristics of peptides. water solubility, resistance to digestion, toxicity, allergenicity and IC\(_{50}\) were carried out. Solubility in water was predicted using PepCalc, which is available at http://pepcalc.com. Gastrointestinal digestion was predict using PeptideCutter, available at http://web.expasy.org/peptide_cutter. Chymotrypsin-low specificity, chymotrypsin-high specificity, pepsin (pH 1.3), pepsin (pH > 2), and trypsin enzymes were used to measure resistance [31]. Toxicity and allergenicity were assessed using bioinformatics tools ToxinPred and AllerTOP which is available at http://www.imtech.res.in/raghava/toxinpred/ and http://www.pharmfac.net/allertop. For the toxicity prediction, support vector machine (SVM) based prediction method was chosen where 0.0 was threshold value [32]. Predicted IC\(_{50}\) was estimated using AHTpin available https://webs.iiitd.edu.in/raghava/ahtpin/index.php. AHTpin is an in \textit{silico} tools which helps to predict, screen and design particularly antihypertensive peptides [38]. Quantitative structure activity relationship (QSAR) based regression models are the basement for the inhibitory
activity estimation of tiny (di and tri) peptides in AHTpin [39]. This basement is acquiescence with Organization for Economic Co-operation and Development (OECD) principles [40]. AHTpin express the IC_{50} value as pIC_{50} value (=-log (IC_{50} x10^6)) to minimize the scale [39].

3. Results and discussion

3.1 Sequences of selected protein

Table 2 lists the identified protein with their accession number. Number of the amino acid presented in selected 14 proteins were between 161 to 577. The dominant proteins were phycerythrin, phycocyanin.
and allophycocyanin that consist of 161 to 164 amino acids and their molecular weights were 17.62 to 17.95 kDa. Whereas, protein alpha-1,4-glucan lyase, isozyme 5 have the highest number of amino acid (577) and the molecular weights of 65.51 kDa.

3.2 The prospect of G. changii as precursor for bioactive peptides

BIOPEP-UWM database gives the number of bioactive peptides expected to be released from the proteins. Based on the existing information in BIOPEP-UWM database (as of 3rd August 2021, 4325 peptides formed in 56 bioactivities have been existed), fragments with 16 biological activities were found in 14 different G. changii proteins.

For total frequency of bioactive peptides occurrence ($\sum A$), phycocyanin alpha subunit and solute carrier protein had the highest value (both gave $\sum A$ of 1.482) followed by allophycocyanin alpha subunit ($\sum A$ 1.472) (table 3). The total frequency of occurrence, $\sum A$ 1.482 of phycocyanin alpha subunit consists of $A_{\text{ACE inhibitor}}$ of 0.463 and $A_{\text{DPP IV inhibitor}}$ of 0.586. This indicates that the main portion of the frequency of occurrences comes from ACE and DPP IV inhibitor. The occurrence varies from 0.688 to 0.586 (DPP IV inhibitor) and 0.518 to 0.336 (ACE inhibitor) for the different proteins. The total occurrences differ 1.129 to 1.482 and similar observation detected regarding the major portions of each protein. Thus, ACE and DPP IV inhibitors were the major part of the occurrence of bioactive fragments and the study focused on these activities. However, no comparison can be made as no study has been reported on in-silico of Gracilaria sp. protein to produce bioactive peptides.

Table 3. The frequency of occurrence of peptides with an assumed activity (A) in particular protein sequences.

| Proteins                                      | NCBI Accession no. | Number of Activities | $\sum A$ | $A_1$  | $A_2$  |
|-----------------------------------------------|--------------------|----------------------|---------|-------|-------|
| Phycoerythrin alpha subunit                   | 50657774           | 14                   | 1.396   | 0.494 | 0.628 |
| Allophycocyanin alpha subunit                 | 51209969           | 14                   | 1.472   | 0.472 | 0.590 |
| Putative NAD-myo-inositol Dehydrogenase       | 4325275            | 14                   | 1.200   | 0.447 | 0.544 |
| LysR transcriptional regulator                | 30409180           | 15                   | 1.399   | 0.445 | 0.640 |
| Sulfate ABC transporter protein               | 51209989           | 15                   | 1.407   | 0.423 | 0.664 |
| Solute carrier protein                        | 4325249            | 14                   | 1.482   | 0.518 | 0.688 |
| Hypothetical protein                          | 11466615           | 15                   | 1.359   | 0.434 | 0.606 |
| Phycocyanin alpha subunit                     | 51210030           | 16                   | 1.482   | 0.463 | 0.586 |
| Alpha-1,4-glucan lyase, isozyme 5            | 5689734            | 16                   | 1.470   | 0.457 | 0.678 |
| ALA dehydratase                               | 13560094           | 15                   | 1.375   | 0.442 | 0.641 |
| Ribulose bisphosphate carboxylase large chain| 730477             | 17                   | 1.461   | 0.467 | 0.656 |
| Gamma-tubulin                                 | 5901583            | 15                   | 1.129   | 0.355 | 0.655 |
| C-type cytochrome biogenesis protein          | 51209870           | 16                   | 1.340   | 0.336 | 0.664 |
| Serine acetyltransferase                      | 6594273            | 16                   | 1.438   | 0.478 | 0.648 |
Among the predicted bioactivity present in the proteins were ACE inhibition, antioxidative, DPP-IV inhibition, DPP-III inhibition and stimulating fragments (table 4). BIOPEP-UWN database also gave

Table 4. Number of potential bioactive peptides and potential biological activity (B) of identified proteins using BIOPEP.

| Protein                                              | NCBI Accession no | Number of active fragments | Stimulating |
|-------------------------------------------------------|-------------------|-----------------------------|-------------|
| Phycoerythrin alpha subunit                           | 50657774          | 81 (0.0204)                 | 4           |
| Allophycocyanin alpha subunit                         | 51209969          | 76 (0.0156)                 | 12          |
| Putative NAD-myoinositol dehydrogenase                | 4325275           | 96 (0.0418)                 |             |
| LysR transcriptional regulator                       | 30409180          | 135 (0.0132)                | 12          |
| Sulfate ABC transporter protein                       | 51209989          | 107 (0.0196)                | 19          |
| Solute carrier protein                                | 4325249           | 113 (0.0239)                | 9           |
| Hypothetical protein                                  | 11466615          | 76 (0.0643)                 | 8           |
| Phycoerythrin alpha subunit                           | 51210030          | 75 (0.0595)                 | 4           |
| Alpha-1,4-glucan lyase, isozyme 5                    | 5689734           | 261 (0.0130)                | 15          |
| ALA dehydratase                                       | 13560094          | 133 (0.0163)                | 9           |
| Ribulose bisphosphate carboxylase large chain         | 730477            | 228 (0.0192)                | 13          |
| Gamma-tubulin                                         | 5901583           | 146 (0.0104)                | 28          |
| C-type cytochrome biogenesis protein                  | 51209870          | 146 (0.0238)                | 47          |
| Serine acetyltransferase                              | 6594273           | 192 (0.0145)                | 15          |

Among the predicted bioactivity present in the proteins were ACE inhibition, antioxidative, DPP-IV inhibition, DPP-III inhibition and stimulating fragments (table 4). BIOPEP-UWN database also gave
the potential activities (B). It was found that ACE inhibitor and DPP IV inhibitor were the maximum released biological active fragments. DPP IV inhibitor had the highest active fragments (95-387) compared to ACE inhibitor fragments (81-192) though potential bioactivity (B) value was higher for the ACE inhibitor. It was also found that different species of Gracilaria family have proteins with various bioactive peptides mainly ACE inhibitor, antioxidative, DPP IV inhibitor etc. Proteins from the identical family expected to be possessed similar bioactive peptides [13,37,38]. The findings are consistent with [39] whereby via in vitro analysis, four seaweeds (S. binderi, P. sulcata, T. conoides and H. macroloba) have been reported to release DPP IV inhibitor. However, G. salicornia extract gave different finding whereby it was a potential source of antibacterial peptide in in vitro and in silico studies [36].

DPP IV inhibitors is one of the major bioactivities of peptides in the seaweed proteins. DPP-IV (EC 3.4.1.4,5), a serine protease is a dipeptidyl aminopeptidase with specificity for cleaving X-Pro or X-Ala dipeptides from N terminal [40]. DPP IV creates lack of insulinotropic activity by reducing gastric repressive peptide and glucagon-like peptide [42]. Consequently, inhibition of DPP IV interest has a healing impact on type 2 diabetes [43]. Diabetes is a long-lasting metabolic disorder which accountable to high blood sugar levels over a long period. Type 2 diabetes mellitus (T2DM) is the major form of diabetes caused ineffective use of insulin. Diabetes, nowadays one of the leading causes of death worldwide. International Diabetes Federation (IDF), projected that about 425 million people were suffering from diabetes globally in 2017 and the number will be 642 million by 2040 (http://www.diabetesatlas.org). However, synthetic DPP-IV drugs are accustomed to have gastrointestinal hostile effects, allergic reactions, skin-related side effects and musculoskeletal disorders [43]. Macro algae can be a valuable natural sources of DPP-IV inhibitory peptides as it has been isolated from many sepsis such as Palmaria palmate, Porphyra columbina, Porphyra dioica and Spirulina platensis [44–46]. In vitro study showed that G. opuntia possessed significant α-amylase, α-glucosidase, and dipeptidyl peptidase-4 (DPP-4, IC50 0.09 mg/mL) inhibitory activities [47]. Besides, many DPP-IV inhibitory peptides have been identified in the enzymatic hydrolysates form several food proteins, including milk proteins [48], rice bran [49], amaranth proteins [50], oat [51], and fish proteins [52,53].

ACE inhibitor is another major bioactivity in the seaweed proteins. Because of its action in the renin-angiotensin system (RAS) and the kallikrein-kinin system, ACE is vital in regulating. Angiotensin I is converted to the active vasoconstrictor angiotensin II by ACE in the RAS. As a result, ACE indirectly raises blood pressure by constricting blood vessels.

Inhibition of ACE activity is a key target for hypertension prevention [54]. Captopril and enalapril are two ACE inhibitors that are commonly used in the treatment of cardiovascular disorders. They do, however, frequently induce adverse effects like coughing, rashes, and taste abnormalities. Synthetic medications can be replaced with natural ACE inhibitory peptides. The N-terminus of ACE-inhibitory peptides is frequently made up of hydrophobic (proline) and aliphatic (isoleucine and leucine) amino acids. [55]. Enzymatic hydrolysis of Porphyra columbina by product protein showed antihypertensive activities with >35% of ACE inhibitor [44]. Spirulina also has been reported to exhibit ACE inhibitory activity [56,57].

3.3 In silico proteolysis of G. changii protein for the production of bioactive peptides
Phycobiliproteins are a group of fluorescent proteins that account for up to half of the total protein content in red seaweeds. [58]. Phycobiliproteins comprise of phycoerythrin, phycocyanin, allophycocyanin, and phycocyanthocyanin [59]. To investigate the potential bioactive peptides released by enzyme actions, three protein sequences were selected (table 5). In the BIOPEP-UWM database, there are 33 types of enzymes, but in this study, three proteases (papain, ficin and stem bromelain) were chosen for the in-silico proteolysis as they are commercially available and plant protease. In-silico analysis was performed by using ‘Enzyme action’ tool of BIOPEP-UWM. Degree of hydrolysis (DH) was between 41.1% to 57.5%. For all the selected protein stem bromelain gave the highest DH (56.52 % – 57.50 %). The release of peptide was not proportionate with the release of bioactive peptide. From wheat gluten, bovine muscle proteins, patatin (potato tuber protein), and quinoa, papain has been shown to successfully synthesize ACE inhibitor and antioxidative peptides. [42]. Previous in silico study on
Quinoa and soybean proteins showed that stem bromelain gave highest DH [60]. In vitro study showed that ACE-1 inhibitor and renin inhibitory peptides can be released by hydrolysis of papain in green algae *Ulva lactuca* [61].

### Table 5. The predicted efficiency of release of bioactive fragments from selected *Gracilaria changii* protein by *in silico* proteolysis.

| Protein                           | Number of Amino Acid in sequence | Enzymes                | DH (%) | ACE Inhibitor | DPP IV Inhibitor |
|-----------------------------------|----------------------------------|------------------------|--------|---------------|------------------|
|                                   |                                  |                        |        | A_E           | W                |
| Phycoerythrin alpha subunit       | 164                              | Papain                 | 41.72  | 0.085         | 0.173            |
|                                   |                                  | Ficin                  | 41.10  | 0.043         | 0.087            |
|                                   |                                  | Stem Bromelain         | 57.05  | 0.073         | 0.148            |
|                                   |                                  |                        |        | 0.079         | 0.073            |
|                                   |                                  |                        |        | 0.126         | 0.117            |
| Allophycocyanin alpha subunit     | 161                              | Papain                 | 43.13  | 0.062         | 0.132            |
|                                   |                                  | Ficin                  | 45.0   | 0.062         | 0.132            |
|                                   |                                  | Stem Bromelain         | 57.5   | 0.062         | 0.132            |
|                                   |                                  |                        |        | 0.081         | 0.137            |
| c-phycocyanin subunit             | 162                              | Papain                 | 44.72  | 0.074         | 0.160            |
|                                   |                                  | Ficin                  | 44.09  | 0.025         | 0.053            |
|                                   |                                  | Stem Bromelain         | 56.52  | 0.074         | 0.160            |
|                                   |                                  |                        |        | 0.111         | 0.189            |

### Table 6. Bioactive peptides predicted to be released from *Gracilaria changii* protein based on *in silico* proteolysis.

| Enzyme                     | ACE inhibitors | DPP-IV inhibitors |
|----------------------------|----------------|-------------------|
| Papain                     | 36             | 43                |
| AF (2), G (10), AR (5),    | AD (2), AE (2),|                    |
| ASL (1), D (1), KF (1), PL (1),| AF (2), AG (10), AT (2),| |
| PT (2), QT (1), YG (1),    | SX (1), YL (2) |                  |
| V (1), VR (1)              |                |                  |
| Ficin                      | 20             | 38                |
| AF (1), AG (1), AR (2),    | AF (1), AG (1),|                    |
| DY (3), NG (1), PG (2), PL (1),| AS (4), AY (1),| |
| QT (2), QT (1), TG (1),    | DR (2), ES (1),|                    |
| V (1), VK (1), VV (1)      | MK (3), MR (1),|                    |
| Stem Bromelain             | 34             | 43                |
| CF (1), DA (3), EA (6),    | DR (2), EG (1),|                    |
| EV (1), IA (4), IG (1),    | HS (1), IA (4),|                    |
| KA (1), KF (1), NG (1),    | KA (1), KF (1),|                    |
| PG (2), PL (1), PR (1),    | KS (1), MQ (1),|                    |
| QT (2), QT (1), YA (1),    | NA (3), NG (1),|                    |
| YG (2), YL (3), YV (1)     | NL (1), NT (1),|                    |
|                            | PF (1), PG (2),|                    |
|                            | PL (1), PS (2),|                    |
|                            | PT (2), QA (2),|                    |
|                            | QT (1), QY (1),|                    |
|                            | TG (1), TK (1),|                    |
|                            | TL (2), TR (1),|                    |
|                            | TS (1), VG (1),|                    |
|                            | VK (1), VL (1),|                    |
|                            | VR (1), VY (1),|                    |
|                            | WY (1)         |                  |
The assessment criteria (AE and W) of DPP-IV and ACE inhibitory peptides were shown in table 5. The release frequency of occurrence of DPP IV inhibitory peptides (0.068 – 1.111) was higher than ACE inhibitory peptides (0.025 – 0.085). Similarly, the relative frequency of release of fragments with assumed activity by selected enzymes (W) was higher in DPP IV inhibitor (0.116 – 0.189) than ACE inhibitor (0.053 - 0.173). Papain showed the better or equal AE and W for the same sequence except DPP IV inhibitory peptides of c-phycocyanin a subunit.

Table 6 shows the predicted DPP-IV and ACE inhibitory peptides to be released from G. changii proteins by in silico proteolysis. These peptides are already in the database. Most of the reported peptides with the given activities were di peptides, except for a reported tripeptide of ASL. There were still not described amply of bioactive peptides. As for the bioactivity of the unidentified peptides, additional study is required. This study focused on the tripeptides. In-silico proteolysis showed that papain and stem bromelain were relatively potential to be released of bioactive peptides. Furthermore, novel peptides were screened for their potentiality.

3.4 Virtual screening and characterization of novel peptide
The in-silico analysis in this study showed that the sequences of all DPP IV inhibitors consisted of only two amino acids. To investigate the novel peptide, ACF, DEW, AYV, DMS, DYT, YCL (papain), DMS, VTY, EMY, AMQ, PTY (ficin), NYS, DMS, PDV, YCL (stem bromelain) were unreported predicted tripeptides to be released by the activity of specific enzymes. According to the PeptideRanker score ACF and YCL are most potential peptides. Scores for the other peptides were <0.5 (0.056 – 0.445). Both peptides were poorly soluble to water due to their hydrophobic residue and not resistance to digestion which is expected for their potentiality (table 7). However, this issue can be overcome through encapsulation [62]. Both peptides were predicted to be non-allergen and non-toxic. Their IC50 value (ACF= 4.97 ìM and YCL=5.65 ìM) showed it is predicted to be potential (table 7). Virtual screening for the novel of Larimichthys crocea protein showed that HGR (His-Gly-Arg) possesses ACE inhibitory activity, with an IC50 value of 106 ± 1.35 μM [63]. Besides Ulva lactuca (AM, PVGCL, PLPP, GPPSP, PKPPAL, GTF), Caulerpa sp. (PWG, FR, IFG, PG, LG, LY), C. sorokiniana (VPL, WG, LA, IR, PG, VY, ICP, RR, PR, VP, PL) reported several peptides [42,61,64]

4. Conclusion
This study revealed that the most potential bioactivity from G. changii proteins were DPP-IV and ACE inhibitors. In silico enzymatic proteolysis showed that stem bromelain and papain both are potential for the releasing of DPP-IV and ACE inhibitory peptides, Furthermore, two novel biopeptides (ACF, YCL) were identified through virtual screening. This study shows that G. changii proteins could be a potential source of bioactive peptides.
Acknowledgements
The authors acknowledge the financial support from the Ministry of Higher Education Malaysia through the Fundamental Research Grant Scheme (FRGS/1/2018/WAB01/UMT/02/4) to complete this research.

References
[1] McHugh D J 2003 A guide to the seaweed industry
[2] FAO 2016 The State of World Fisheries and Aquaculture
[3] Ireland C M, Copp B R, Foster M P, Mcdonald L A and Radisky D C 1993 Biomedical Potential of Marine Natural Products Mar. Biotechnol. 11–43.
[4] Kim S and Wijesekara I 2010 Development and biological activities of marine-derived bioactive peptides: A review J. Funct. Foods 2 1–9.
[5] Liu M, Wang Y, Liu Y and Ruan R 2016 Bioactive peptides derived from traditional Chinese medicine and traditional Chinese food: A review Food Res. Int. 89 63–73.
[6] Qi H, Zhao T, Zhang Q, Li Z, Zhao Z and Xing R 2005 Antioxidant activity of different molecular weight sulfated polysaccharides from Ulva pertusa Kjellm (Chlorophyta) J. Appl. Phycol. 17 527–34.
[7] Freile-pelegrin Y, Robledo D, Chan-bacab M J and Ortega-morales B O 2008 Antileishmanial properties of tropical marine algae extracts Fitoterapia 79 374–7.
[8] Roufik S, Å S F G and Turgeon S L 2006 In vitro digestibility of bioactive peptides derived from bovine b-lactoglobulin Int. Dairy J. 16 294–302.
[9] Wijesinghe W A J P and Jeon Y 2012 Enzyme-assistant extraction (EAE) of bioactive components: A useful approach for recovery of industrially important metabolites from seaweeds: A review J. Appl. Phycol. 83 6–12.
[10] Cian R E, Garzón A G, Ancona D B, Guerrero L C and Drago S R 2015 Hydrolyzates from Pyropia columbina seaweed have antiplatelet aggregation, antioxidant and ACE I inhibitory peptides which maintain bioactivity after simulated gastrointestinal digestion LWT - Food Sci. Technol. 64 881–8.
[11] Cao D, Lv X, Xu X, Yu H, Sun X and Xu N 2017 Purification and identification of a novel ACE inhibitory peptide from marine alga Gracilaria lemaneiformis protein hydrolysate Eur. Food Res. Technol. 243 1829–37.
[12] Sun S, Xu X, Sun X, Zhang X, Chen X and Xu N 2019 Preparation and identification of ACE inhibitory peptides from the marine macroalg Ulva intestinalis Mar. Drugs 17 1–17.
[13] Lee H A, Kim I H and Nam T J 2015 Bioactive peptide from Pyropia yezoensis and its anti-inflammatory activities Int. J. Mol. Med. 36 1701–6.
[14] Qu W, Ma H, Pan Z, Luo L, Wang Z and He R 2010 Preparation and antihypertensive activity of peptides from Porphyrha yezoensis Food Chem. 123 14–20.
[15] Udenigwe C C 2014 Bioinformatics approaches, prospects and challenges of food bioactive peptide research Trends Food Sci. Technol. 36 137–43.
[16] Minkiewicz P, Dziuba J, Iwaniak A, Dziuba M and Darewicz M 2008 BIOPEP Database and Other Programs for Processing Bioactive Peptide Sequences J. AOAC Int. 91 965–80.
[17] Garg S, Apostolopoulos V, Nurgali K and Mishra V K 2018 Evaluation of in silico approach for prediction of presence of opioid peptides in wheat J. Funct. Foods 41 34–40.
[18] Agirbasli Z and Cavas L 2017 In silico evaluation of bioactive peptides from the green algae Caulerpa J. Appl. Phycol. 29 1635–46.
[19] Lin K, Zhang L, Han X, Meng Z, Xin L, Gong P and Cheng D 2018 Yak milk casein as potential precursor of angiotensin I-converting enzyme inhibitory peptides based on in silico proteolysis
Food Chem. 254 340–7.

[22] Fu Y, Feveile J, Marie M and Lametsch R 2016 Revalorisation of bovine collagen as a potential precursor of angiotensin I-converting enzyme (ACE) inhibitory peptides based on silico and in vitro protein digestions J. Funct. Foods 24 196–206.

[23] Gangopadhyay N, Wynne K, Connor P O, Gallagher E, Brunton N P, Rai D K and Hayes M 2016 In silico and in vitro analyses of the angiotensin-I converting enzyme inhibitory activity of hydrolysates generated from crude barley (Hordeum vulgare) protein concentrates Food Chem. 203 367–74.

[24] Udenigwe C C 2015 Towards rice bran protein utilization: In silico insight on the role of oryzacystatins in biologically-active peptide production Food Chem. 30 3–6.

[25] Chan P T and Matanjun P 2017 Chemical composition and physicochemical properties of tropical red seaweed, Gracilaria changii Food Chem. 221 302–10.

[26] Chen P T, Matanjun P, Yasir S and Tan T S 2014 Antioxidant and hypolipidaemic properties of red seaweed, Gracilaria changii J. Appl. Phycol. 26 987–97.

[27] Benjama O and Masniyom P 2012 Biochemical composition and physicochemical properties of two red seaweeds (Gracilaria fisheri and G. tenuistipitata) from the Pattani Bay in Southern Thailand Songklanakarin J. Sci. Technol. 34 223–30.

[28] Gressler V, Sumie N, Toyota M, Colepicolo P, Mancini J, Pavan R and Pinto E 2010 Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algal species Food Chem. 120 585–90.

[29] Hong D D, Hien H M and Son P N 2007 Seaweeds from Vietnam used for functional food, medicine and biofertilizer J. Appl. Phycol. 19 817–26.

[30] Matanjun P and Mohamed S 2009 Nutrient content of tropical edible seaweeds, Eucheuma cottonii, Caulerpa lentillifera and Sargassum polycystum J. Appl. Phycol. 21 75–80.

[31] Wng P, Tan L, Nawi H and Abubakar S 2006 Proteomics of the red alga, Gracilaria changii (gracilariales, rhodophyta) J. Phycol. 42 113–20.

[32] Lafarga T, Connor P O and Hayes M 2014 Peptides Identification of novel dipeptidyl peptidase-IV and angiotensin-I-converting enzyme inhibitory peptides from meat proteins using in silico analysis Peptides 59 53–62.

[33] Hall F, Johnson P E and Liceaga A M 2018 Effect of enzymatic hydrolysis on bioactive properties and allergenicity of cricket (Gryllodes sigillatus) protein Food Chem. 262 39–47.

[34] Mooney C, Haslam N J, Pollastri G and Shields D C 2012 Towards the Improved Discovery and Design of Functional Peptides: Common Features of Diverse Classes Permit Generalized Prediction of Bioactivity PLoS One 7.

[35] Wu J, Aluko R E and Nakai S 2006 Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship modeling of peptides containing 4-10 amino acid residues QSAR Comb. Sci. 25 873–80.

[36] Ruiz J Á G, Ramos M and Recio I 2004 Angiotensin-converting enzyme-inhibitory activity of peptides isolated from Manchego cheese. Stability under simulated gastrointestinal digestion Int. Dairy J. 14 1075–80.

[37] Nongonierma A B, Mooney C, Shields D C and Fitzgerald R J 2014 In silico approaches to predict the potential of milk protein-derived peptides as dipeptidyl peptidase IV (DPP-IV) inhibitors Peptides 57 43–51.

[38] Tahir R A, Bashir A, Yousaf M N, Ahmed A, Dali Y, Khan S and Sehgal S A 2020 In Silico identification of angiotensin-converting enzyme inhibitory peptides from MRJP1 PLoS One 15 1–18.

[39] Gramatica P 2007 Principles of QSAR models validation: Internal and external QSAR Comb. Sci. 26 694–701.

[40] Kumar R, Chaudhary K, Singh Chauhan J, Nagpal G, Kumar R, Sharma M and Raghava G P S 2015 An in silico platform for predicting, screening and designing of antihypertensive peptides Sci. Rep. 5 1–10.
[41] Hildebrandt M, Reutter W, Arck P, Rose M and Klapp B F 2000 A guardian angel: the involvement of dipeptidyl peptidase IV in psychoneuroendocrine function, nutrition and immune defence Clin. Sci. 99 93–104.
[42] Zeng Z, Luo J, Zuo F, Zhang Y and Ma H 2016 Screening for potential novel probiotic Lactobacillus strains based on high dipeptidyl peptidase IV and α-glucosidase inhibitory J. Funct. Foods 20 486–95.
[43] Liu R, Cheng J and Wu H 2019 Discovery of Food-Derived Dipeptidyl Peptidase IV Inhibitory Peptides: A Review Int. J. Mol. Sci. 20 463.
[44] Cian R E, Martínez-Augustin O and Drago S R 2012 Bioactive properties of peptides obtained by enzymatic hydrolysis from protein byproducts of Porphyra columbina Food Res. Int. 49 364–72.
[45] Harney P A, O’Keeffe M B and Fitzgerald R J 2015 Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga Palmaria palmata Food Chem. 172 400–6.
[46] Cermeño M, Stack J, Tobin P R, O’Keeffe M B, Harney P A, Stengel D B and Fitzgerald R J 2019 Peptide identification from α: Porphyra dioica protein hydrolysate with antioxidant, angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory activities Food Funct. 10 3421–9.
[47] Makkar F and Chakraborty A 2017 Antidiabetic and anti-inflammatory potential of sulphated polygalactans from red seaweeds Kappaphycus alvarezi and Gracilaria opuntia Int. J. Food Prop. 20 1326–37.
[48] Uchida M, Ohshiba Y and Mogami O 2011 Novel Dipeptidyl Peptidase-4 – Inhibiting Peptide Derived From β-Lactoglobulin J. Pharmacol. Sci. 117 63–6.
[49] Hatanaka T, Inoue Y, Arima J, Kumagai Y, Usuki H, Kawakami K, Kimura M and Mukaihara T 2012 Production of dipeptidyl peptidase IV inhibitory peptides from defatted rice bran Food Chem. 134 797–802.
[50] Velarde-salcedo A J, Barrera-pacheco A, Lara-gonzález S, Montero-morán G M, Díaz-gois A, González E, Mejia D, Barba A P and Rosa D 2013 In vitro inhibition of dipeptidyl peptidase IV by peptides derived from the hydrolysis of amaranth (Amaranthus hypochondriacus L.) proteins Food Chem. 136 758–64.
[51] Bleakley S, Maria H, Nora O, Eimear G and Tomas L 2017 UsPredicted Release and Analysis of Novel ACE-I, Renin, and DPP-IV Inhibitory Peptides from Common Oat (Avena sativa) Protein Hydrolysates Using in Silico Analysising in Silico Analysis in Silico Analysis foods 6 108.
[52] Huang S, Jao C, Ho K and Hsu K 2012 Peptides Dipeptidyl-peptidase IV inhibitory activity of peptides derived from tuna cooking juice hydrolysates Peptides 35 114–21.
[53] Sila A, Alvarez O M, Haddar A, Frikha F, Dhulster P, Nedjar-Aroume N and Bougatat A 2016 Purification, identification and structural modelling of DPP-IV inhibiting peptides from barbel protein hydrolysate J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1008 260–9.
[54] Shahidi F and Zhong Y 2008 Bioactive Peptides Journa AOAC Int. 91 914–31.
[55] Lee S Y and Hur S J 2017 Antihypertensive peptides from animal products, marine organisms, and plants Food Chem. 228 506–17.
[56] Lu J, Ren D F, Xue Y L, Sawano Y, Miyakawa T and Tanokura M 2010 Isolation of an antihypertensive peptide from alcalase digest of spirulina platensis J. Agric. Food Chem. 58 7166–71.
[57] Heo S, Ko S, Kim C S U, Oh G, Ryu B, Qian Z J I, Kim G, Park W O N S U N, Choi I, Phan T T V Y, Heo S, Kang D, Yi M and Jung W 2017 A heptameric peptide purified from Spirulina sp. gastrointestinal hydrolysate inhibits angiotensin I-converting enzyme- and angiotensin II-induced vascular dysfunction in human endothelial cells Int. J. Mol. Med. 39 1072–82.
[58] Niu J F, Wang G C, Zhou B C, Lin X Z and Chen C S 2007 Purification of R-phycocerythrin from Porphyra haitanensis (Bangiales, Rhodophyta) using expanded-bed absorption J. Phycol. 43 1339–47.
[59] Denis C, Ledorze C, Jaouen P and Fleurence J 2009 Comparison of different procedures for the extraction and partial purification of R-phycoerythrin from the red macroalga Grateloupia turuturu Bot. Mar. 52 278–81.

[60] Guo H, Richel A, Hao Y, Fan X, Everaert N, Yang X and Ren G 2020 Novel dipeptidyl peptidase-IV and angiotensin-I-converting enzyme inhibitory peptides released from quinoa protein by in silico proteolysis Food Sci. Nutr. 8 1415–22.

[61] Garcia-vaquero M, Mora L and Hayes M 2019 In Vitro and In Silico Approaches to Generating and Identifying Angiotensin-Converting Enzyme I Inhibitory Peptides from Green Macroalga Mar. Drugs 17 204.

[62] Homayouni A, Azizi A, Ehsani M R, Yarmand M S and Razavi S H 2008 Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream Food Chem. 111 50–5.

[63] Yu Z, Wu S, Zhao W, Ding L, Li J and Liu J 2019 Virtual screening and molecular docking for exploring ACE inhibitory peptides in Larimichthys crocea nebulin protein Int. Food Res. J. 26 1417–26.

[64] Tejano L A, Peralta J P, Yap E E S, Panjaitan F C A and Chang Y W 2019 Prediction of bioactive peptides from chlorella sorokiniana proteins using proteomic techniques in combination with bioinformatics analyses Int. J. Mol. Sci. 20.