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In Silico Bioactive Peptide Prediction from The Enzymatic Hydrolysates of Edible Seaweed Rubisco Large Chain

Ayse Kose[1, *]

[1]Ege University Faculty of Engineering Department of Bioengineering, 35100, Bornova, Izmir, Turkey.

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Corresponding Author
Tel.: +902323115811
E-mail: ayse.kose@ege.edu.tr

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Abstract

Seaweeds are one of the ancient food supplements on Earth. Especially Asian countries use seaweeds as the fundamental ingredient in their cuisine. Seaweeds are photosynthetic organisms living in aquatic ecosystems and in the coastal territories. Seaweeds out of farm areas are frequently observed as coastal wastes. However, seaweeds are outstanding sources for bioactive substances and investigation bioactive properties of seaweed RuBisCO has never been done. RuBisCO is the most abundant protein on Earth but a vast amount of RuBisCO goes through waste. In this study, bioactive peptide prediction of frequently consumed seaweed RuBisCO proteins were analyzed *in silico* to identify possible bioactive peptides as substitute or support for grain, meat, and dairy based bioactive peptides. A huge portion of peptides were di-, tri- peptides with IC_{50} values less than 300 µM according to the comparison of BIOPEP database. Including gastric digestion, more than half of the peptides showed DDP-IV and ACE inhibitory activity followed by antioxidant properties. Also, novel anti-inflammatory and anti-cancer peptides were found through *in silico* analysis.

Introduction

Proteins have been major food supplement on human nutrition. Humans consume proteins from grains like wheat, rice, beans, lentil, chickpea, soy, meat products from poultry, aquaculture or red meat industry and dairy products like milk, yoghurt, cheese, whey etc. which are rich in protein content (FitzGerald et al., 2019). These sources of proteins are valuable because the cryptic bioactive peptides are embedded in mother protein and only shows intrinsic activities when the mother protein is degraded via enzymatic processes through digestive system (Fan et al., 2014).

Bioactive peptides obtained from marine, plant, dairy and meat sources show various bioactivities such as antioxidant, antihypertensive, immunomodulatory, anticancer, anti-diabetic (Yanhong Li et al., 2008; Yunliang Li et al., 2015; Tejano et al., 2019; Udenigwe et al., 2017a). The health benefits and their potential on providing a healthy diet and potential to prevent chronic diseases made them a new attraction for functional nutrition and therapeutics (Onuh et al., 2014). Besides, they are derived from natural sources, reliable and sustainable supplements comparing them with synthetic origins. Idea of taking nutritional supplements then medicine is also a more positive image considering

the psychological choices of the individuals (Kose & Oncel, 2015b).

Bioactive peptides are 2-20 amino acid chain length short oligopeptides (Morris et al., 2008; Wijesekara et al., 2011). They can be synthesized synthetically, produced by recombination techniques (Liang et al., 2014) or obtained from various extraction methods like chemical, enzyme treatment and fermentation (Udenigwe et al., 2017a). Exogenous and endogenous peptidases and proteases can serve to decode these bioactive peptides through natural digestion, fermentation or other industrial processes which are mild process conditions compared to other physical techniques (Udenigwe et al., 2013).

The bioactivity of a peptide is highly dependent on the length and combination of the amino acids which defines their molecular mass, solubility and interaction at aqueous environment, net charge on the peptide and most importantly bioactivity to be defined (Agyei et al., 2018; Udenigwe et al., 2013). If enzymatic hydrolysis is going to be used, the choice of the enzyme is also an important parameter to optimize. Each enzyme has a various recognition site to cut the protein. This affects the total length, molecular mass, amino acid sequence and other physicochemical and bioactivity characteristics of the peptides in the mixture. As novel pharmaceutical compounds, peptides catch attention as dietary medicine due to low toxicity and their significant function (Zhou et al., 2013). Recently, sports food, special nutrition supplements contain bioactive peptides after understanding outstanding bioactivities of peptide hydrolysates.

Macroalgae, also known as seaweeds, has been one of the main food supplements in Asian countries; Japan, Korea, China, Philippines being the leaders on mass production and consumption. There are various species known with high nutritional values currently available even local markets. The physiochemical properties, protein, carbohydrates, pigment, vitamin, mineral and fiber content attribute to a balanced nutrition. The protein content is changeable, brown seaweeds are usually low in protein yield in comparison to green and red seaweeds. As a local and traditional food source the bioactive peptides inside seaweeds are of importance for deciphering cryptic bioactive peptides.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, E.C. 4.1.1.39) is one of the mostly expressed enzymes various autotrophic organisms as algae (microalgae and seaweeds), bacteria (photosynthetic and chemoautotrophic bacteria, cyanobacteria, archaea) and plants (Udenigwe et al., 2017a). RuBisCO is responsible from the catalytically conversion of CO_2 to organic carbon sources in Calvin-Benson Cycle (Udenigwe et al., 2017a). The primary sequence of the RuBisCO known to be conserved among species, but it is also visible that the amino acid composition and length of the protein may show difference which can be considered as a clue for evolutionary adaptations. RuBisCO is directly related to crop yield, photosynthetic efficiency, mineral and water usage of the organisms and the major contributor of the global carbon cycle. RuBisCO is documented to have bioactive peptides for the treatment of various chronic illnesses from cardiovascular to neurodegenerative diseases and oxidative stress related physiological malfunctions (Selvaraj et al., 2017). It is one of the most abundant proteins on Earth (Udenigwe et al., 2017a). RuBisCO consumption thus probability of consumed bioactive peptides derived from RuBisCO is also higher as well along with diet as naturally.

Computational tools can be applied to mining bioactive peptides from various sources (Udenigwe et al., 2013). Common webservers are developed for *in silico* peptide prediction and mostly angiotensin converting enzyme (ACE), dipeptidyl peptidase (DPP)-IV and antioxidant peptides could be deciphered from various sources rich in protein (Gu et al., 2011; Lafarga et al., 2015; Nongonierma & Fitzgerald, 2014). Udenigwe et al. (2013) showed DPP-IV, ACE, and antioxidant activities of several RuBisCO proteins from common cereals utilized. Selveraj et al. (2017) showed the bioactive peptide prediction from commercially edible microalgae species RuBisCO. However, even though seaweeds are also a major food sources there is no data for *in silico* peptide prediction considering seaweeds as protein sources especially RuBisCO utilization in functional food industry is highly demanding.

The aim of this study is to draw a scheme using computational *in silico* tools for novel peptide prediction from most consumed edible seaweed species using RuBisCO as mother protein. Combination of bioactive peptides, distribution, predicted bioactivity, anticancer and toxic properties are computed using ExPASy, BIOPEP, UniProt, ToxinPred, iCAP, iACP, AntiCP, MLACP and Bioware webservers. The anti-inflammatory peptide prediction was done using AIPred. The study is valuable output for further synthesis of synthetic analogs of bioactive peptides or recombinant production for the detection of actual bioactivities.

Materials and Methods

Sequences of Seaweed RuBisCO

Edible seaweeds; *Undaria pinnatifida*, *Sargassum fusiforme*, *Fucus vesiculosus*, *Rhodymenia palmate*, *Ulva lactuca*, *Laminaria japonica* (*Sargassum japonica*), *Mastocarpus stellatus* (*Gigartina stellata*), *Ulva intestinalis* (*Enteromorpha intestinalis*), *Gracilaria edulis*, *Porphyra umbilicalis*, *Callophyllis variegate* species were selected according to their consumption and biotechnological importance. RuBisCO large subunit accession numbers were obtained from UniProtKB/Swiss-Prot and TrEMBL databases for primary amino acid sequences for further analysis (Table 1). RuBisCO large Chain (rbcL) sequences were used only

because there were not enough data for all the species RuBisCO Small Chain (rbcS).

Multiple Sequence Alignments (MSAs)

The amino acid concentration from each protein was calculated using ProtParam Tool (http://web.expasy.org/protparam/) to compute physicochemical properties of RuBisCO proteins through amino acid sequences. Multiple sequence alignments (MSAs) were done with ClustalW2 (http://www.clustal.org/clustal2/) and phylogenetic tree was constructed using Clustal Omega. Seaweed RuBisCO sequence alignments were done in comparison to several conventional edible plants *Oryza sativa* (P0C512), *Hordeum vulgare* (P05698), *Triticum aestivum* (P11383), *Zea mays* (P00874) *Avena sativa* (P48684) RuBisCO large chain (Udenigwe et al., 2013).

***In Silico* Analysis**

The enzymatic hydrolysis was achieved using BIOPEP Web server (http://www.uwm.edu.pl/biochemia/index.php/en/biopep). The database provides enzymatic hydrolysis, bioactivity prediction and comparison of possible achieved peptides in their databases (Minkiewicz P. et al., 2008). The frequency of occurrence of susceptible bioactive peptides in seaweed RuBisCO was calculated using BIOPEP. Industrially utilized proteases such as thermolysin (EC 3.4.24.27) and papain (EC 3.4.22.2) were chosen for *in silico* peptide prediction from enzyme(s) action of BIOPEP web server. The crude protein hydrolysates were analyzed for their possible bioactivities such as ACE inhibition, antioxidative capacity, DPP-IV inhibition, and so on based on the calculated bioactivity prediction scores.

Due to common utilization of these seaweeds as food sources, simulated gastric digestion was also done in BIOPEP server. Simulated Gastrointestinal (GI) digestion was carried out for RuBisCO proteins as well. Protein and peptide sequences were evaluated for potential cleavage by GI enzymes (pepsin, pH = 1.3), trypsin (EC 3.4.21.4) and chymotrypsin (EC3.4.21.1). The potential bioactivities of the peptides were analyzed using PeptideRanker and peptide score was calculated using 0.5 as threshold (http://bioware.ucd.ie/~compass/biowareweb/).

The probable toxic peptides were predicted using ToxinPred, http://www.imtech.res.in/raghava/toxinpred/ (Gupta et al., 2013). Anticancer peptide prediction was done according to iACP (Chen et al., 2016), AntiACP (Tyagi et al.,2013) and MLACP (http://thegleelab.org/MLACP.html) databases. Peptides giving 0.5 and higher probability on Peptide Ranker further analyzed for anti-inflammatory prediction using AIPred (http://thegleelab.org/AIpred.html).

Results and Discussion

Investigation of Seaweed RuBisCO

RuBisCO is correspondent to a major portion of protein in plants and algae and still is the most abundant protein on Earth (Udenigwe et al., 2017b). The increasing attention on empowering rural economies also effects the algae cultivation and investments. Development of seaweed-based industries is a part of circular economy where sustainable utilization of existing resources becoming an urgent topic day by day due to increasing crisis on food and fuel debate. Algae as a major food supplement and food ingredient spreads from Asia to Europe and other parts of the world as a new hype oriental food source (Fleurence et al., 2018). Increase in the consumption of algae species makes them a major attention for novel functional food search. Thus, the bioactive peptide encrypted in the seaweed is of importance.

Table 1. List of seaweeds utilized for *in silico* peptide determination

UniProt Accession Number	Number of amino acid residues	Protein size (kDa)	Theoretical PI	Grand Average of Hydropathy (GRAVY)	Organisms	Traditional name
A0A220NUZ6	488	48.08	5.58	-0.034	*Sargassum fusiforme*	Hijiki
A0A0R6M8Y2	488	54.07	5.71	-0.079	*Undaria pinnatifida*	Wakame
Q2PQH5	488	53.94	5.52	-0.105	*Fucus vesiculosus*	Bladderwrack
Q9THF8	488	54	5.54	-0.141	*Rhodymenia palmata*	Dulse
Q7Y838	445	49.06	6.26	-0.283	*Ulva lactuca*	Sea lettuce
J7F8P2	488	54.04	5.70	-0.091	*Laminaria japonica (Sargassum japonica)*	Sweet kelp
Q32632	488	54.19	5.70	-0.123	*Mastocarpus stellatus (Gigartina stellata)*	Carrageen moss
Q6X4L8	451	49.72	6.04	-0.245	*Ulva intestinalis (Enteromorpha intestinalis)*	Hollow green nori
Q2TVU7	485	53.77	5.98	-0.122	*Gracilaria edulis*	-
Q760R7	488	53.97	5.80	-0.133	*Porphyra umbilicalis*	Purple laver
H6TIB2	476	52,80	5.65	-0.100	*Callophyllis variegata*	Carola

The seaweed species were selected with respect to their high consumption. For example, *Undaria pinnatifida*, *Sargassum fusiforme*, *Porphyra umbilicalis* and *Ulva* species are commonly consumed in Asian countries as Japan being one of the leaders. On the other hand; species like *Palmaria* is mostly common in Western countries such as Denmark, Britain, Iceland (Mouritsen et al., 2013). The seaweed species were chosen throughout their mass consumption among the world as an oriental cuisine and novel food ingredients for vegetarian/vegan nutrition. Development of the marine ecosystem for bio-economy is an urgent topic since the idea of farming doesn't corresponds to land plants but aquatic environments are also included. Since rapid increase in the global population is a risk for food crisis; nutritious and functional food contents are motivated. With the developments in peptide-based biosciences, functional peptide-based additives are formulated for food applications as to regenerate, repair or enhance the current health status.

Bioprospecting of a bioactive peptide from mother protein source is an inconvenient and long path. Even tough existing physical treatment methods are helpful and promising for novel peptide (s) discovery, tracing back to mother protein source and predict the origin of the peptide is a challenging work when considered larger scale applications. Results of enzymatic hydrolysis of a protein depends on the choice of enzyme, operation conditions and purification methods. When a bulk protein content such as whole cells (Kose et al., 2015) are treated with enzymes, it is challenging to decipher original protein codes of the bioactive peptides unless there is not a well-defined proteome map is not available. Thus, *in silico* peptide prediction tools allow researchers to screen possible peptides from large protein sequence information. *In silico* hydrolysis and peptide bioactivity prediction has an algorithm to hydrolysis the known protein sequence to individual peptides considering the enzyme cut sequences. Also, screening comparative analysis of peptides and protein sources, identifying homology and activity is another interactive branch of peptidomics. Therefore, combining bioactive peptide prospecting using *in silico* design tool and databases could give rapid and reliable results and has the potential to draw a line from prediction to function.

Uniprot accession numbers along with the traditional name was presented in Table 1. RuBisCO Large chain (RbcL) was used in this study because there were not RbcS sequence data for all the species. Besides, RbcL is majority of the protein because the activity of RuBisCO is low; large quantities of the enzyme are produced to continue cellular functionalities. The RbcL protein was in between 54-48 kDa and there was not significant difference on their molecular masses.

Sequence alignment of RuBisCO proteins in selected seaweeds was done to see the homology in between the proteins. Cereals RuBisCo such as *Oryza sativa* P0C512, *Hordeum vulgare* P05698, *Triticum aestivum* P11383, oat *Avena sativa* P48684, *Zea mays* P00874 were also aligned with seaweeds to see their phylogenetic relations. Ulva species showed a great relativeness with cereals however the other seaweeds species were not closely related as Ulva represented (Figure 1). As in the plant RuBisCO, seaweed RuBisCO could show secondary structure homology however their primary amino acid sequence showed significant differences. The phylogeny tree confirmed their far relations. The species were diversified with their protein sequences which were an advantage to decipher more bioactive peptides. Sequence homology could give an opportunity to have an increased chance of having similar peptides in large quantities however also having diverse sequence alignments could be utilized as the possibility to have various bioactive peptides never characterized before. Since RuBisCO is an untapped source of protein (having half of the soluble proteins in plant leaves) it is advantageous to work with various types of RuBisCO origins. In this case seaweed possess a great opportunity considering aquatic mass production could be an advantage over plants however in the case of plants, there are increasing number of plant wastes which could be a starting material for RuBisCO bioactive peptides.

The total amino acid sequences were collected from Uniprot data, and amino acid distribution was investigated with Expasy Protparam tool. Amino acids with aromatic side chains showing nonpolar characteristics tend to show more antioxidant and radical scavenging activity, such as Trp, Phe, Val, Leu, Ile, Ala and Arg (Yanhong Li et al., 2008; Schurink, 2007) When amino acid composition was analyzed; it was found that Ala, Gly, Leu, Ile, Val were the major amino acids in the proteins followed by Thr, Arg, Asp, Glu, Lys (Figure 2). The amino acid distribution displayed that major contribution of amino acids were also amino acids responsible for ACE and DDP-IV inhibitory amino acids like Gly, Leu, Ile (Lafarga et al., 2014, 2016). The composition of these amino acids was as Gly (8-11%), Leu (8-9%), Trp (6%), Ile (4-6%), Tyr (4-5%). Phe (4%) and Pro (3-4%). The results could be concluded as the peptides derived from seaweed RuBisCO proteins could represent ACE, DPP-IV inhibitory activities along with antioxidant capacities. The bioactivity of the peptide is dependent on molecular masses, amino acid sequences, order of amino acids and structures of N-terminal, C-terminal amino acids. For example, in the case of tyrosinase inhibitory peptides existing of at least one Arg residue in combination with Val, Phe, Leu, Ala could be strong inhibitors for skin whitening properties (Schurink et al., 2007). Also C-terminal Tyr residue peptides containing Arg, Val, Ala, Phe, Cys also show strong binding properties for tyrosinase enzymes in various enzyme inhibition models (Abu Ubeid et al., 2009; Ochiai et al., 2016). Mostly bioactive peptides have a molecular mass less than 3kDa and strong candidates are smaller than 1500 Da. C-terminal amino acid residue could be critical for ACE inhibitory as well. Aromatic amino acids

Table 2. The frequency of occurrence (A) of cryptic bioactive peptide sequences of seaweed RuBisCO proteins according to BIOPEP Database

Activity	A0A0R6M8Y2	A0A220NUZ6	Q2PQH5	Q9THF8	Q7Y838	J7F8P2	Q32632	Q6X4L8	Q2TVU7	Q760R7	H6TIB2
ACE Inhibitors*	0.4734	0.4772	0.4775	0.4283	0.5034	0.4713	0.4508	0.5166	0.4536	0.4324	0.4517
Ubmp Activating**	0.0205	0.0183	0.0205	0.0143	0.0180	0.0205	0.0102	0.0200	0.0144	0.0123	0.0147
Alpha-Glucosidase Inhibitor***	0.0020	0.0023	0.0020	0.0020	0.0045	0.0020	0.0020	0.0044	0.0041	0.0020	0.0021
Antiamnestic***	0.0102	0.0091	0.0102	0.0082	0.0090	0.0102	0.0082	0.0089	0.0082	0.0082	0.0084
Antioxidative*	0.0738	0.0822	0.0799	0.0635	0.0854	0.0758	0.0656	0.0887	0.0701	0.0738	0.0714
Antithrombotic***	0.0102	0.0091	0.0102	0.0082	0.0090	0.0102	0.0082	0.0089	0.0082	0.0082	0.0084
DPP-IV Inhibitor*	0.6455	0.6484	0.6516	0.6393	0.6180	0.6496	0.6619	0.6208	0.6722	0.6537	0.6828
Hypotensive**	0.0225	0.0228	0.0246	0.0225	0.0292	0.0225	0.0205	0.0266	0.0247	0.0205	0.0210
Immunomodulating***	0.0041	0.0046	0.0041	0.0020	0.0045	0.0041	0.0020	0.0044	0.0021	0.0041	0.0021
Inhibitor***	0.0020	0.0023	0.0020	0.0041	0.0022	0.0020	0.0041	0.0022	0.0062	0.0041	0.0063
Neuropeptide***	0.0082	0.0091	0.0082	0.0102	0.0045	0.0082	0.0123	0.0044	0.0062	0.0102	0.0084
Regulating**	0.0164	0.0137	0.0164	0.0102	0.0180	0.0184	0.0143	0.0022	0.0124	0.0164	0.0147
Stimulating**	0.0328	0.0297	0.0328	0.0246	0.0337	0.0328	0.0389	0.0177	0.0330	0.0287	0.0294

* Peptides having major bioactivity, **Peptides having mild bioactivity, ***Peptides having no significant activity

Table 3. A values of seaweed RuBisCO after simulated Gastric Digestion (GD), Papain and Thermolysin hydrolysis on BIOPEP

Function	A0A220NUZ6	A0A0R6M8Y2	Q2PQH5	Q9THF8	Q7Y838	J7F8P2	Q32632	Q6X4L8	Q2TVU7	Q760R7	H6TIB2
Gastric Digestion (GD)											
ACE Inhibitor	0.0494	0.0485	0.0514	0.0336	0.0434	0.0494	0. 0375	0.0450	0.0457	0.0415	0.0407
Antioxidative	0.0079	0.0044	0.0059	0.0020	0.0065	0.0079	0.0040	0.0064	0.0040	0.0040	0.0061
Stimulating	0.0040	0.0044	0.0040	0.0020	0.0043	0.0040	0.0020	0.0043	0.0060	0.0020	0.0061
DPP- IV Inhibitor	0.0494	0.0441	0.0494	0.0336	0.0521	0.0474	0.0415	0.0535	0.0040	0.0395	0.0020
Renin Inhibitor	0.0040	0.0044	0.0040	0.0040	0.0043	0.0040	0.0040	0.0043	0.0477	0.0040	0.0467
DPP- III Inhibitor	0.0099	0.0088	0.0079	0.0119	0.0087	0.0099	0.0119	0.0086	0.0139	0.0119	0.0142
DH_t%	36.44	36.20	36.64	35.25	35.87	36.24	36.24	35.84	36.85	36.44	36.05
Papain											
Antioxidative	0.0020	0.0066	0.0059	0.0040	0.0022	0.0040	0.0039	0.0021	0.0080	0.0060	0.0040
ACE Inhibitor	0.0415	0.0463	0.0435	0.0514	0.0325	0.0375	0.0453	0.0300	0.0517	0.0476	0.0445
DPP- IV Inhibitor	0.0613	0.0683	0.0593	0.0810	0.0629	0.0593	0.0748	0.0578	0.0815	0.0774	0.0850
Hypotensive	0.0020	0.0022	0.0020	0.0020	0.0043	0.0020	0.0020	0.0043	0.0020	0.0020	0.0020
DH_t%	40.39	41.06	40.00	39.80	42.83	40.20	38.07	43.13	40.24	36.96	38.74
Thermolysin											
Antioxidative	0.0079	0.0088	0.0079	0.0040	0.0390	0.0079	0.0040	0.0407	0.0298	0.0040	0.0040
ACE Inhibitor	0.0336	0.0330	0.0336	0.0514	0.0043	0.0336	0.0316	0.0043	0.0596	0.0316	0.0324
DPP- IV Inhibitor	0.0514	0.0617	0.0534	0.0810	0.0477	0.0514	0.0593	0.0471	0.0060	0.0593	0.0020
DH_t%	38.82	39.29	38.62	37.62	35.00	38.81	38.42	36.05	38.25	38.22	38.54

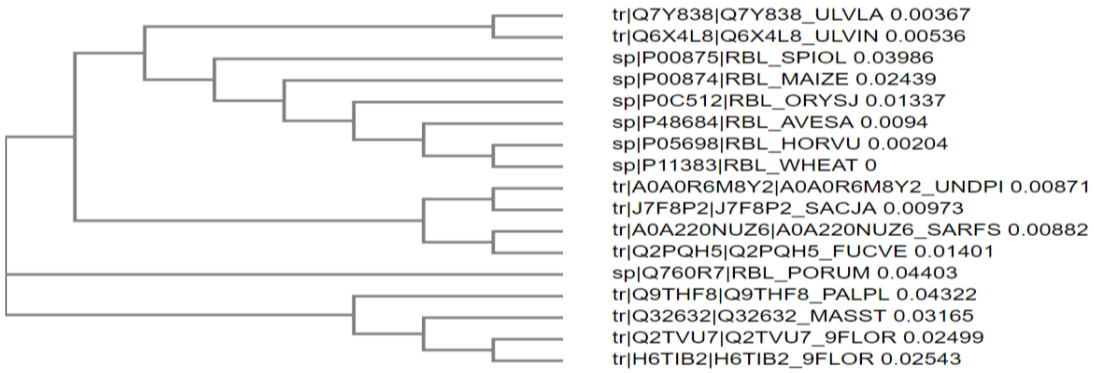

Figure 1. Multiple sequence alignment of Seaweed RuBisCO and cereals

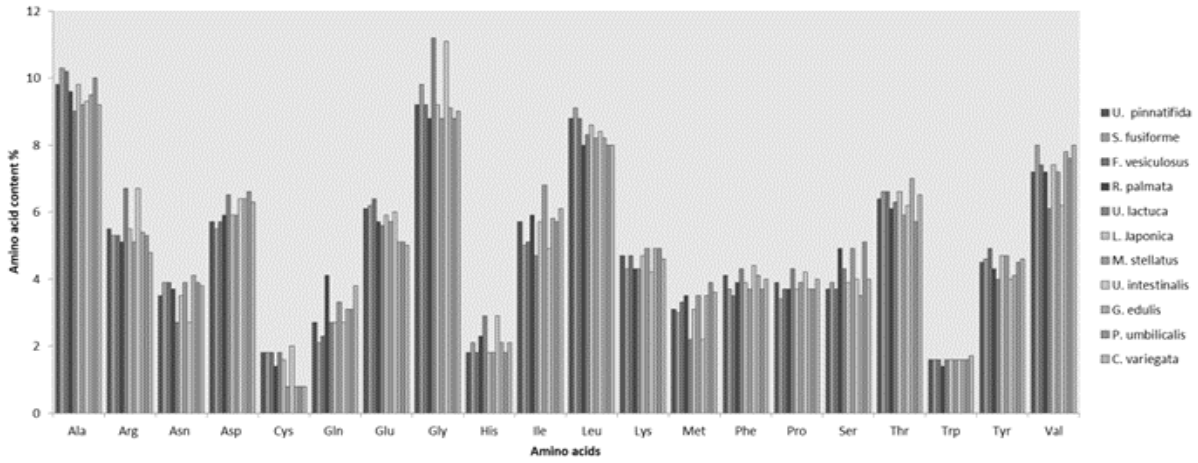

Figure 2. Amino acid sequences of seaweed RuBisCO

as Trp, Tyr and Phe and aliphatic amino acids Ile, Ala, Leu and Met give strong correlation with ACE inhibitory activity however N-terminal residue doesn't contribute significantly to the activity (J. Li et al., 2018). In the case of DPP-IV inhibitory N-terminal Trp could be responsible from the certain activity. Ile-Pro-Ile analog structures could show strong inhibitory activity because Pro existence in the peptide could contribute inhibition characteristics (Liu et al., 2019). AS a result of preliminary amino acid analysis, as it was reported before, RuBisCO from seaweed also hold a great opportunity for generating various bioactive peptides.

Bioactivity Prediction from Seaweed RuBisCO Proteins

The frequency of occurrence (A) values of the proteins is highly related with the amino acid sequence of the mother protein. A values were calculated with BIOPEP database. A=a/N formula represents "a" as the number of bioactive peptides in the mother protein and N is the total amino acid residues (Minkiewicz P. et al., 2008). A value is helpful to predict the bioactivity segment of the protein and thus it is also utilized as a guide to decide which proteolytic enzyme to use to obtain peptide fragments. Prior to any enzymatic hydrolysis prediction, A values were obtained from seaweed RuBisCO proteins (Table 2). The major predicted bioactivities showed strong DPP-IV and ACE inhibition capacity. DPP-IV and ACE inhibitory activities were not significantly diverse in between the species; however, for ACE inhibitory activity *U. intestinalis* was higher with A value of 0.5166 and for DPP-IV inhibition activity *C. variegate* showed highest value as 0.6828. The other potential activities could be utilized were followed as antioxidative, stimulating, UbMP activating, regulating and hypotensive. A values for other bioactivities such as antithrombotic and antiamnesic (Table 2) were so low that the attention for obtaining bioactive peptides from seaweed RuBisCO could be given for ACE inhibitory, DDP-IV inhibitory and antioxidant activities. The results are in a good accordance with the literature and demand in the bioactive ingredient industry (Selvaraj et al., 2017; Udenigwe et al., 2017a).

***In Silico* Enzymatic Hydrolysis**

BIOPEP web server has an "Enzyme(s)" action to simulate proteolysis by endopeptidases introduced in the database. By choosing the enzyme of interest, a protein sequence can be hydrolyzed into peptide products. The system allows to hydrolyze the proteins

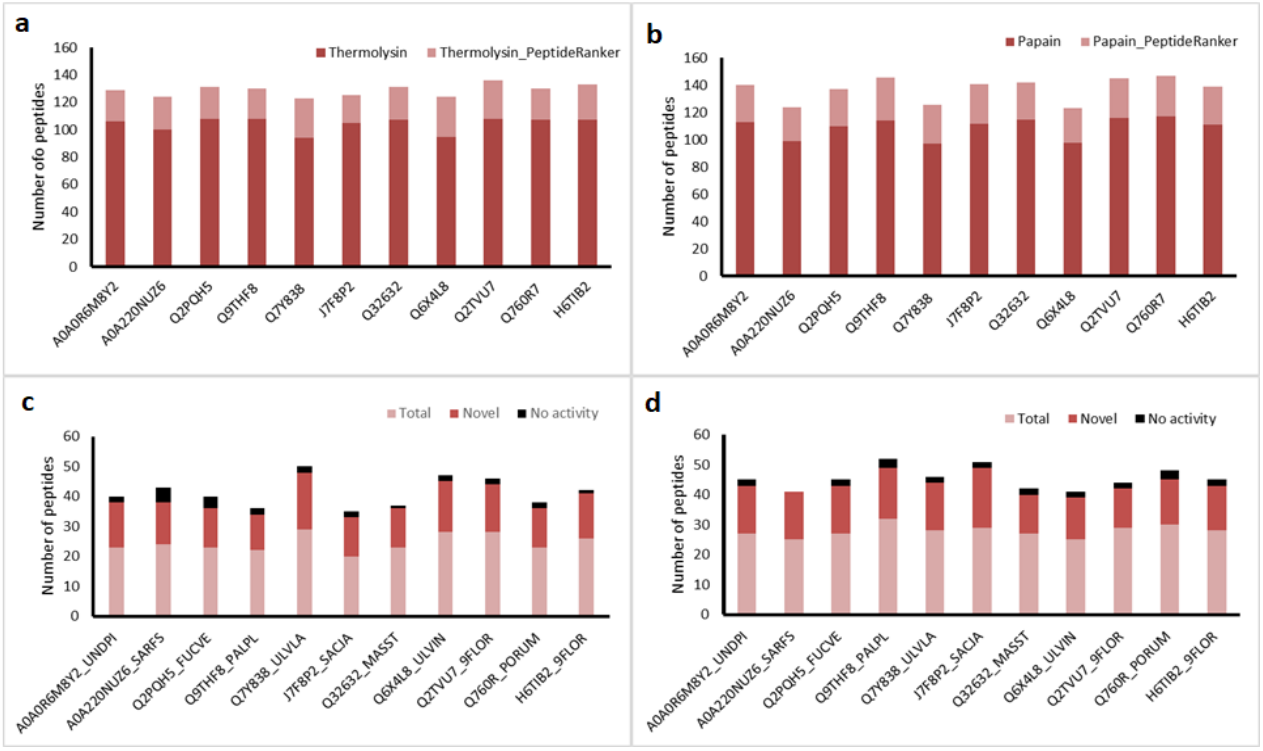

Figure 3. Number of peptides released from a) thermolysin, b) papain hydrolysis, number of peptides having Peptide Ranker score higher than 0.5 for c) thermolysin, d) papain.

with three enzymes simultaneously. This is beneficial to simulate gastric digestion profiles. In this study, Thermolysin and papain enzymes were selected. The seaweed RuBisCO proteins were digested *in silico* by thermolysin and papain using BioPEP. The both enzymes are conventional food processing enzymes and could give a strong contribution for the development of predicted bioactive peptides. These enzymes also give peptides 2-50 amino acid chains which increase possibility to find short sequence low molecular weight bioactive peptides. The A values showed that both enzymes give mostly DPP-IV and ACE inhibitory peptides followed by antioxidative properties (Table 3). Some hypotensive and neuropeptide scores could be observed however, due to low A values, these bioactivities could be neglected. Regardless from the enzyme, di- and tri-peptides were released from mother proteins for each seaweed species. More than 600 peptides could be observed. Because seaweed species share common conserved sequences, the digestion results gave almost the same dipeptides.

The digested peptides were evaluated on PeptideRanker to find the possible peptides with certain bioactivities. 0.5 in PeptideRanker was taken as threshold and number of the peptides in Thermolysin and Papain were calculated (Figure 3). For thermolysin 69 possible bioactive peptide was calculated including dipeptides and decapeptides. 81 different peptides were identified with papain digestion. 11 of these peptides were obtained both papain and thermolysin digestion. Seven of these peptides were identified with ACE and/or DPP-IV inhibitor activities in BIOPEP database; MR (DPP-IV inhibitor), VW (ACE, DDP-IV inhibitor, antioxidant, glucose uptake inhibitor), IPG (ACE inhibitor), APG (DPP-IV inhibitor), AG (DDP-IV and ACE inhibitor), YR (DPP-IV inhibitor) and IG (ACE inhibitor). On the other hand, even though the PeptideRanker score was under 0.5; some dipeptides with ACE and DPP-IV inhibitory activities could be identified from thermolysin and papain digestion. The resulting peptides possess some bioactivities and deciphering them could be another topic of mining bioactive peptide from natural sources.

Simulated GI Digestion

Simulated GI digestion for seaweed RuBisCO proteins was done to understand the occurrence of cryptic bioactive peptides in digestive tract. One of the major sources of observing bioactive peptides is the GI digestion. Naturally, when humans and animals consume protein sources, proteinaceous materials meet with GI enzymes such as amylase, lipases, pepsin, trypsin, and chymotrypsin. Direct consuming of protein rich foods is the major and cheaper way to obtain bioactive peptides and predict their beneficial effects rather than taking specially designed peptide formulations unless it is not recommended for health practitioners for people in need. For this purpose, BIOPEP enzymes action tool was utilized for GI simulation. Each protein is digested *in silico* with pepsin (pH > 1.3), trypsin (EC 3.4.21.4) and chymotrypsin (EC3.4.21.1).

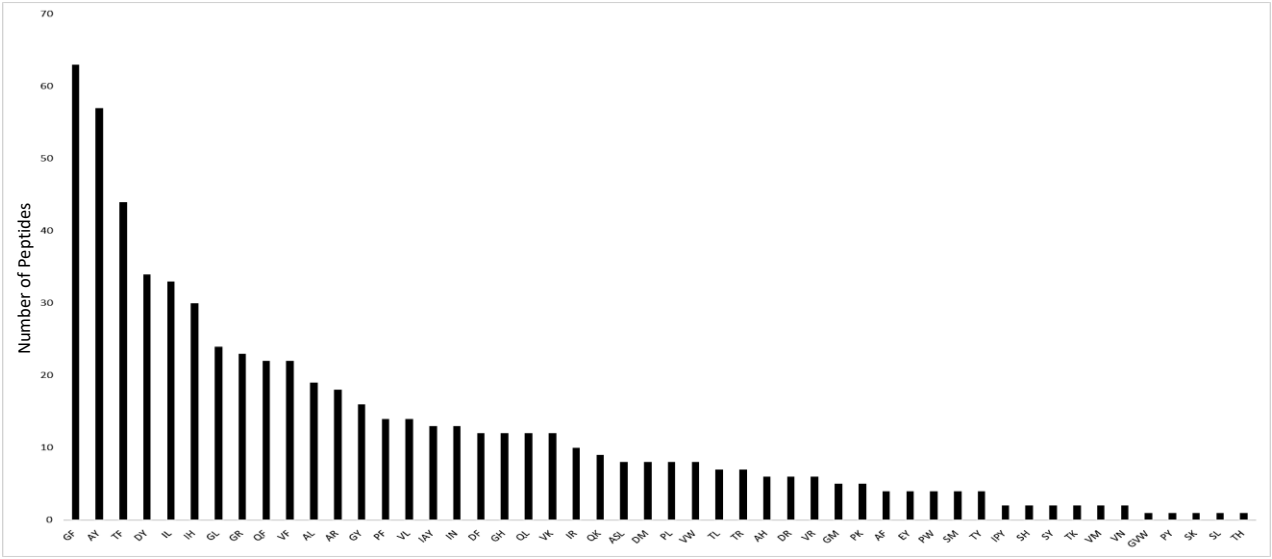

Figure 4. Number of relased bioactive peptides and their sequences in seaweed RuBisCO proteins.

GI digestion simulation resulted that when these proteins are consumed as food supplement, they are more susceptible as ACE inhibitory and DDP-IV inhibitory activities followed by antioxidant capacity (Table 3). Digestion resulted on mostly dipeptides and several tripeptides. Number of obtained peptides for each protein was approximately 30 and most of them already existed in the peptide database from several sources. From overall gastric digestion of seaweed RuBisCO, 50 different di- and tripeptides were found with primary activity of ACE and DPP-IV inhibitory followed by DPP-III inhibition and antioxidant effect. GF, AY, IH, IL, TF occurred more than 30 times due to the rich composition of seaweed proteins in terms of Gly, Phe, Ala and Ile amino acids which mostly give antioxidant, ACE ad DPP-IV inhibitory activities to the peptides (Figure 4). Peptides like VF, YL, FY, AF, VR,SY,KY, AR, TF, IAY, AY, ASL, IL, IPY, IH and IPY with IC_{50} values less than 300 µM (Chang & Alli, 2012) peptides were abundant also in seaweed peptides when compared to food sources like legumes, grains, meat and dairy products.

Inhibition of DPP-IV enzyme is new type of therapeutic approach on the treatment of type 2 Diabetes mellitus (Deng et al., 2018). Peptides such as TF, GF, IPY, AY with multiple bioactivities is advantage considering gastric digestion could help releasing peptides with multifunctionalities. ACE inhibitors function as ACE receptor blockers, usually people having hypertension use drugs containing ACE inhibitors. ACE inhibitor molecules are also important due to strong relation of cardiovascular diseases with hypertension problems. The demand on natural peptides with ACE inhibitory activities are increasing, firstly peptides could be derived from food sources naturally and by a regular diet. This study showed that, seaweeds holds a great opportunity for dietary ACE and DPP-IV inhibitory peptides as results observed by simulated GI digestion. The limitation here could be the second digestion of an oligopeptide when consumed by diet, however hence we showed most of the ACE and DPP-IV inhibitory peptides are di- and tripeptides having a low chance to digested by GI tract.

Opioid Peptides

Food derived peptides, and proteins have cryptic opioid peptides with some analgesic and anxiolytic effect. Milk and wheat derived peptides were known to have some opioid properties, however; RuBisCO large subunit of spinach gave 2 new opioid peptides binding the D1 receptor, activating σ1 to show some anxiolytic effects (Hirata et al., 2007). These opioid peptides also helped impairing cognitive behavior in mice. Rubiscolin-6 was also found to be a stimulating peptide for food intake in mice model. The rubiscolin-5 (YPLDL) and rubiscolin-6 (YPLDLF) were found in *Ulva lactuca* and *Ulva intestinalis*. The other seaweed RuBisCO proteins were not having rubiscolins in their mother amino acid sequence. When *U. lactuca* and *U. intestinalis* RuBisCO was BLAST, the sequence similarity was found to be 88-98% in most of the microalgae species which are biotechnologically important such as *Chlorella* sp., *Chlorella vulgaris*, *Chlorella elipsoidea*, *Dunaliella*, *Ostreococcus tauri,* and rubiscolins were conserved. We blast *U. lactuca* and *U. intestinalis* RuBisCO against Spinach RuBisCO large chain and found 86.07% and 86.86% sequence similarities, respectively. Especially the sequences of 15 amino acids which embedded rubiscolin peptides were highly conserved when compared to spinach. According to *in-silico* digest, there was no enzyme cutting the exact sequence of rubiscolin-5/6 however, some of the enzymes cut rubiscolins in specific locations to give other bioactive fragments. The abundant portion was an ACE inhibitory peptide IAY (IC_{50}=12.59 µM) and obtained by enzymes such as chymotrypsin A/C, chymase, ficin, metridin, subtisilin and cathepsin G. ACE and DDP-IV inhibitory peptide PL (IC_{50}=337.32 µM) was obtained by gastric enzymes,

papain, ficin, subtisilin, chymase, metridin, bromelain, calpain 2, cathepsin G, leukocyte elastase, oligopeptidase F. Another bioactive dipeptide was YP (ACE inhibitory IC50=720 µM; DPP-IV inhibitory IC50=3170µM). Yang et al. showed the pepsin digestion of spinach natural RuBisCO and synthetic peptide sequence (ICYVAYPLDLFEEG) where rubiscolins exist. In Ulva species, Rubiscolin sequences are conserved but V is replaced with I and C was replaced with A. When simulated pepsin digestion was done, it was susceptible that -AYPLDL- sequence can be released depending on the enzymatic hydrolysis conditions (Udenigwe et al., 2017a). This gives the idea that oral administration of Ulva species could result as the intake of opioid rubiscolins. The concentration of the rubiscolin is however dependent on the degree of hydrolysis, intake in GI tract and total consumption of Ulva biomass on daily basis. When rubiscolin oral administration was done to mice, the activity of the peptide was considered as a critical point for the receptor-ligand interactions to signal increasing food intake (Kaneko et al., 2012).

Anti-Inflammatory (AI) Peptide Prediction

Anti-inflammatory responses of host organism are a strong and complex defense systems to fight against pathogens and unwanted substances. Development of novel anti-inflammatory molecules is urgent for curing auto-immune and inflammatory chronic diseases (Yuan et al., 2016). Synthetic and natural compounds could have certain AI activity. Recently peptides derived from food, marine and fungal organisms also venom of spiders, snakes and some other arthropods are susceptible sources for natural treatment of chronic and acute inflammation. When an inflammation stimulus is observed cellular signaling molecules as cytokines of the interleukin (IL) families, tumor necrosis factor alpha (TNF-α), prostaglandins (PG), nitric oxide (NO), and leukotrienes (LTs) are released from cells. Also, enzymes such as nitric oxide synthase (iNOS) and protein kinase C (PKC) are crucial for response generation. In this part of the study, peptides derived from gastric digestion, papain and thermolysis hydrolysis which also show a score higher than 0.5 in Peptide Ranker was utilized for AI peptide prediction (Dadar et al., 2019).

Among 80 peptides with various lengths of amino acids we found 24 peptides susceptible of AI activity. Scoring of the peptides probable activity was done according to the prediction server. However, peptides giving score under 0.2 were negative and score between 0.3-0.4 show low probability, 0.4-0.5 show medium probability and 0.5- to higher show higher probability of AI activity. In general, peptides having 5 or more amino acid sequences gave also increasing probability of AI activity. In this case, we found 4 peptides ACDIYR (0.549), QDWVSL (0.549), SVICMIDL (0.545), VICKWMR (0.527). 5 of peptides showed medium AI activity and the rest was predicted to show low AI activity (Table 4). According to the observations both Peptide Ranker and AI databases, the base sequence DMIL looks interesting for the meaning of AI activity. Peptides generated from DMIL such as HDMIL and NDMIL also susceptible from

Table 4. *In silico* Anti-inflamatory peptide prediction

Peptide Sequence	Peptide Ranker Score	Combined RF score (probability)	Allergenity	Tanimoto similarity index with protein in database
ACDIYR	0.64966	0.549	Yes	0.78 / NCBI gi number 51316200
QDWVSL	0.491286	0.549	Yes	0.81/ NCBI gi number 30908930
SVICMIDL	0.513388	0.545	Yes	0.77/ NCBI gi number 30908930
VICKWMR	0.807916	0.527	No	0.74/UniProtKB accession number P80813
YCMEG	0.54467	0.514	Yes	0.83/ NCBI gi number 51316200
CYDIEPL	0.555929	0.451	Yes	0.72/ NCBI gi number 30908930
IHVWHMP	0.677358	0.437	Yes	0.75/ NCBI gi number 30908930
IHCG	0.550607	0.429	Yes	0.82/NCBI gi number 51316200
WKDISF	0.715281	0.421	Yes	0.79/ NCBI gi number 51316200
ACKWSPEL	0.713336	0.406	No	0.70/UniProtKB accession number Q7M1U4
ACKWSP	0.679043	0.384	Yes	0.75/ NCBI gi number 30908930
DPVMI	0.505963	0.379	No	0.83/ UniProtKB accession number P80820
KNDMIL	0.519663	0.376	Yes	0.83/ NCBI gi number 30908930
AIWSR	0.624844	0.367	Yes	0.85/ NCBI gi number 51316200
LYYL	0.593875	0.363	Yes	0.83/ NCBI gi number 51316200
MDKF	0.8092	0.354	Yes	0.83/ NCBI gi number 51316200
YWDPEHVIL	0.49981	0.353	Yes	0.71/ NCBI gi number 25090949
HDMIL	0.616997	0.352	No	0.83/ UniProtKB accession number P80820
DMDW	0.860391	0.348	No	0.82/ UniProtKB accession number P80820
DMIL	0.606999	0.346	No	0.83/ UniProtKB accession number P80820
QPYL	0.685675	0.345	Yes	0.82/ NCBI gi number 51316200
NDMIL	0.605273	0.331	No	0.81/ UniProtKB accession number P80820
ACDL	0.620836	0.325	No	0.83/UniProtKB accession number P80820
YYL	0.598987	0.319	No	0.83/UniProtKB # Q8IZT6

* Probability scores for AIP prediction (> 0.5, high; 0.5 < P < 0.4, medium; <0.3 low)

certain AI activities. L, Y, S, R and E in N-terminal flanking of peptides were susceptible for AI activity in RuBisCO hydrolysates, all the peptides having medium and high activity has one of these amino acids in their N terminal sides. Also, L, Q, S and R at C terminal side give high probability of AI activity. The peptides with high AI prediction show C terminal and N terminal conserved amino acid sequences in the case of ACDIYR and QDWVSL, SVICMILD. Sequences such as C terminal P, G and A and N terminal A, G, V, P and N decreases the probability (Gupta et al., 2017). Peptides showing probability less than 0.4 has these amino acid sequences in their N and C terminal sides. Seaweed RuBisCO derived AI peptides are promising candidates for novel food derived AI-peptides. Thus, further *in vitro* and *in vivo* activity screening of RuBisCO derived AI peptides are required.

Toxic Peptide Prediction

The recent therapeutic approaches are moving towards to peptide/protein-based therapies. Rather than having synthetic analogs, enzymatic hydrolysates rich in susceptible bioactive peptides is one of the main topics in peptide-based therapeutics research. However, peptides still have some concerns on stability, activity, immunogenicity, and toxicity. Cytotoxic effects are mostly examined using *in vitro* animal cell cultures. However, when protein hydrolysates are in the case certain chromatographic purification steps should be adapted to see the active/toxic peptide(s) inside the hydrolysates cocktail. In this study an in-silico toxin prediction tool, ToxinPred, was adapted to mine toxic sequences inside seaweed RuBisCO proteins. The mother protein sequences were digested *in silico* using BIOPEP enzymes tool to decipher the enzymes responsible for the release of certain toxic peptides. 13 different peptide sequences were found in 11 RuBisCO enzymes. *S. fusiforme*, *M. stellatus*, *C. edulis* and *C. variegate* species didn't give any toxic peptides. *P. palmata* had 8 different peptides susceptible from toxicity. Table 5 gives probable toxic peptides derived from seaweed species. The table also shows the release of peptide when digested certain proteolytic enzymes. It is known that peptides containing Cys, His, Asn, Pro have a higher tendency to be toxic (Gupta et al., 2013). Peptides predicted to be o be toxic contains at least one Cys residue except AKMGYWDADY which is released from four different seaweed species. However, another study find that peptides rich in Pro didn't give significant toxic effect (Lafarga et al., 2014). It is considered as not to be a rule of thumb however susceptibility is increasing. The proteolytic degradation properties were analyzed, and enzymes releasing toxic peptides were identified (Table 5). Gastrointestinal enzymes such as pepsin and some commercial proteases such as subtisilin, proteinase K, V-8 protease could release the toxic peptides. It could be noted that gastric digestion of these seaweed species could give some toxic peptides released to GI tract however their toxicity should be investigated *in vitro* and *in vivo* experiments via designing synthetic peptides and purification via enzymatic hydrolysis of bulk protein.

From toxic peptides obtained from ToxinPred database results, anticancer properties of the peptides were also analyzed using iACP, AntiCP, MLACP *in silico* anticancer peptide prediction tools. The results given by each tool gave some different results. All the databases agreed on the anticancer possibility of P4, P5 and P9 and non-anticancer properties of P13. According to antiCP all the peptides except P13 could show anticancer activity. iACP suggested that P4-P8 and P9-10 could be anticancer and MLAPC suggested P2-P5 and P9 could show some anticancer activity. SWM score for common anticancer peptide P9 was the highest (1.32 for AntiCP). These peptides could be candidates for certain anticancer activity however the verification of *in silico* results should be done to see the actual effect *in vitro* and if so, continue for *in vivo* experiments.

The *in-silico* bioactivity prediction tools are helpful to choose the starting point when dealing with mining and bioprospecting approach for the development of natural therapeutics. In this study, RubisCO protein hydrolysates from seaweed species showed that macroalgae was strong candidates for the development of ACE, DPP-IV inhibitory peptides and some additional bioactivities as anticancer and antioxidant properties. The peptides were predicted via in silico enzymatic hydrolysis web server rather than designing in vitro experiments. By this method, the time and experimental design for actual hydrolysis were decreased. However, it should be kept in mind that, one of the limitation of this method s the requirement of known protein sequences. On the other hand, when these sequences are known the method is simple and effective. The activity of the possibly released peptides could be achieved with the verification of designing synthetic peptides of the predicted ones and also recombinant bulk production of the relevant peptide could be an option to develop the industrial aspects of the natural bioactive peptides. *In vitro* and *in vivo* activity models, *in vitro* digestion examination will help the verification of the peptides and validated by various wet-lab techniques.

Conclusion

Seaweed RuBisCO proteins could be promising source for development of DPP-IV, ACE inhibitors and antioxidant activities. According to our results, most of the strong peptide inhibitors (IC_{50}<300μM) for DDP-IV and ACE obtained in dairy and meat sources could be achieved from seaweed species, too. RuBisCO papain and, thermolysin hydrolysates are novel sources of anti-inflammatory peptides as well. Consuming seaweed products could be one of the functional food substitutes for individuals preferring vegetarian and vegan nutrition. However, verification and actualization of the toxicity and bioactivity of the peptides could be done to

Table 5. Toxic peptide prediction and list of proteases releasing toxic sequence

Species	Peptide code	Toxic peptide sequence	SWM Score	Digestive enzyme	Allergen	
P. palmata (Q9THF8)	P1	TACDLYRAKC	0.13	-	No	0.66/UniProtKB accession number P80814
	P2	ACDLYRAKCY	0.53	-	Yes	0.69/ NCBI gi number 280371
	P3	CDLYRAKCYK	0.48	-	Yes	0.66/UniProtKB accession number P01153
	P4	DLYRAKCYKV	0.21	-	No	0.67/UniProtKB accession number Q7M1S9
	P5	LYRAKCYKVD	0.32	V-8 protease	Yes	0.68/NCBI gi number 54036219
	P6	YRAKCYKVDA	0.18	Pepsin Pancreatic elastase II Oligopeptidase F	Yes	0.69/NCBI gi number 248058
	P7	RAKCYKVDAV	0.16	-	Yes	0.65/NCBI gi number 46396597
	P8	AKCYKVDAVP	0.17	Clostripain Prolyl oligopeptidase	No	0.67/UniProtKB accession number P80810
U. lactuca (Q7Y838) **U. intestinalis* (Q6X4L8)	P9	DRYKGRCYDI*	0.06	Pepsin Pancreatic elastase II Leukocyte elastase Oligopeptidase F Lactocepin	Yes	0.68/NCBI gi number 25090949
	P10	RYKGRCYDIE*	0.21	Proteinase K V-8 protease	No	0.63/UniProtKB accession number P80814
U. pinnatifida (A0A0R6M8Y2) *F. vesiculosus* (Q2PQH5) *S. japonica* (J7F8P2) *P. umbilicalis* (Q760R7)	P11	AKMGYWDADY	0.06	-	No	0.71/UniProtKB accession number Q7M1G2
U. intestinalis (Q6X4L8)	P12	NATAGTCEEM	0.01	Pepsin Proteinase K Cathepsin G Metridin Pancreatic elastase Subtilisilin Oligopeptidase F Lactocepin	Yes	0.68/NCBI gi number 54036219
	P13	GDDACLQFGG	0.03	-	No	0.71/UniProtKB accession number Q8IZT6

develop novel formulas. Challenges as cost, public preference, contribution to nature and natural sources, circular economy of the processes should be considered.

Ethical Statement

Ethical statement is not required.

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Author Contribution

The author designed and did the analysis. The manuscript is written and edited by the author.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper."

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