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Peptide Analysis of Protein Extracts from *Caulerpa Lentillifera* by Nano LC-ESI-Ms/Ms and Their Potential as Precursor of Biologically Active Peptides – *In Silico* Approach

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Abstract: Peptidomics research is gradually positioning in food science fields due to an increasing interest in peptides released from food proteins with health benefits and nutraceutical properties. Referable to the complexity of these compounds, their study requires advanced analytical techniques such as tandem mass spectrometry. Seaweed naturally has high nutritional value and many health-promoting effects. Hence, this work aimed to characterize the peptide and to virtual screening for their potential as a precursor of biologically active peptides. The total soluble protein was extracted from *Caulerpa lentillifera* using the phenol ammonium acetate precipitation method. In solution, digestion was carried out using trypsin on 500 µg of protein. Peptide sequencing was accomplished using nano liquid chromatography-electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS), and Peaks Studio 7 was used for the analysis of MS/MS data and *de novo* peptide sequencing using an average local confidence above 90%. The results showed that 76 peptides mapped to selected proteins and 145 were *de novo* peptide sequences. *In silico* approaches of both peptide sequences resulted in 15 types of biological activity characteristics of peptides from among 44 categories as listed in the BIOPEP-UWM database, and motifs with the ACE inhibitory activity occur most frequently. These findings are relevant to the search for bio-functional ingredients as constituents of functional foods or provide added value to nutraceutical foods. Significantly, the methodology described here might apply to discovering the potential in any organism with incomplete genome data.

Keywords: Seaweed, peptidomic, *de novo* sequencing, Nutraceuticals, ACE-inhibitor

Introduction

Proteomics and peptidomic research are progressively positioned in food science due to an increasing interest in protein or peptides released from food proteins with health benefits and nutraceutical properties. Due to the complexity of these compounds, their study requires modern analytical techniques such as tandem mass spectroscopy (MS/MS). Mass spectrometry (MS) and MS/MS are now well-established as potent analytical tools for protein and peptide analysis, possessing high sensitivity and great specificity (Chen, 2008). It also can isolate a single species, including post-translational modification of peptides from a background of thousands of co-existing species. In many cases, amino-acid sequences of 8 to 10 residues carry sufficient information to determine the protein from which the peptide is derived (Wu et al., 2006).

Bioactive proteins and peptides are derived from food and have physiological, hormone-like effects on human organisms. They act directly through their presence in the undisturbed food (Hartmann & Meisel, 2007). Bioactive peptides are encrypted in the protein's primary structure and inactive until released by enzymatic hydrolysis. It can occur during digestion by proteolytic enzymes in the gastrointestinal tract or fermentation and food processing (Udenigwe & Aluko, 2012; Udenigwe & Howard, 2013). Bioactive peptides can exhibit local effects in the gastrointestinal system or cause systemic effects after intestinal absorption and entering the circulatory system. They range in size from 2 to 50 amino acid residues (Hernández-Ledesma et al., 2011). Once

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liberated, the bioactive peptides may act as regulatory compounds with hormone-like activity, exhibiting a wide range of biological functions, such as antihypertensive, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic, immunomodulatory, hypoglycemic, opioid, and antiproliferative activities.

The proteomic analysis of *C. lentillifera* proteins poses two significant difficulties: First, because of the complexity of the matrices examined, it is difficult to obtain high-quality protein extracts, and second, due to the lack of sequenced genomes, the database information available is limited. Therefore, this study used the phenol extraction method because it has been shown to generate high-quality protein extracts from various plant species, including our sample, *C. lentillifera*. Only a few studies have examined the seaweed protein, and no reports have been published regarding *C. lentillifera* proteome or peptides from Sabah, Malaysia waters. Therefore, the characterization of *C. lentillifera* proteins could lead to the discovery of new bioactive molecules, thus increasing the value of this seaweed as a food product.

Method

Sample Preparation

Green seaweed *C. lentillifera* was collected from Semporna, Sabah. The seaweed identification was guardedly confirmed based on morphological characteristics. AcDP was prepared according to the method described by Awang et al. (2010) with some modifications to the first steps to eliminate the non-protein compounds efficiently. This washing step was performed five times, and the resulting polyphenol-free seaweed powder was air-dried. AcDP was stored at -80^{o}C until protein extraction.

Total Soluble Protein Extraction, Quantification and Yield

Phenol extraction methanol-ammonium acetate precipitation extraction of total soluble protein was performed according to Carpentier et al. (2005) and Awang et al. (2010) with little modifications. The total soluble protein in the seaweed samples was determined using the Bradford assay (Bradford, 1976). The total soluble protein concentration was expressed as microgram per microliter (µg/µL), and the protein yield was calculated from this result.

In-Solution Tryptic Digestion, Mass Spectrometry Analysis and Database Searches

For in-solution digestion, approximately 0.5 mg of proteins were reduced, alkylated, and digested with trypsin. After overnight incubation at 37 °C, trypsin activity was stopped with 1 µL of concentrated acetic acid. The samples were later concentrated by vacuum concentrator and kept at -80 °C until use. The samples were dissolved in 0.1% formic acid and filtered. The peptide mixtures were analyzed by on-line nanoflow liquid chromatography using the EASY-nLC II system (Thermo Scientific, San Jose, CA, USA) with 10 cm capillary columns of an internal diameter of 75 µm filled with 3 µm Easy-Column C18-A2 (Thermo Scientific, San Jose, CA, USA) and coupled with pre-column (Easy-Column, 20 0.1 mm i.d., 5 m; Thermo Scientific, San Jose, CA, USA) at flow rate of 0.3 l/min. Running buffers were (A) deionised distilled water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. Instrument control and data recording were performed using Xcalibur ver. 2.1 (Thermo Scientific, San Jose, CA, USA) with a mass tolerance threshold of 5 ppm. Data analysis was performed using PEAKS studio ver. 7.0. PEAKS were used to construct predicted peptide sequences for MS/MS data without referring to the known protein databases. The process produced a list of *de novo* peptide sequences identified from MS/MS spectra. This was followed by database matching to the UniProt-Seaweed database.

In Silico Assessment of Peptides Identified Using BIOPEP-UWM Database

The peptides identified by bioinformatics were searched against the BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia) to find bioactive fragments (motifs) in both identified peptide sequences. (Minkiewicz et al., 2019). This program allows the user to evaluate the peptide sequence profiles of peptide potential with biological activity. Presently, 44 biological activity characteristics of peptides and 2609 motifs of bioactive peptide sequences were listed in the BIOPEP-UWM database. This option acquired BIOPEP ID, name of peptides, potential activity of peptide, number of peptides, and location of bioactive peptide in protein

sequences. Meanwhile, the frequency of occurrence of fragments with given activity (A) in the selected protein was taken as the evaluation parameter and calculated based on equation 1:

$$A = a/N \quad \text{……equation 1}$$

Where a = the number of bioactive peptides and N = the total number of amino acid (AA) residues in the protein chain. In addition, the total frequency of bioactive fragments (∑A) in each of the four sequences was also calculated.

Data Analysis

Data analysis was done by using Microsoft Excel 2016. Data from the BIOPEP-UWM database were placed in Microsoft Excel, where the predicted bioactive peptides and activities were sorted, analysed and tabulated into tables and figures.

Results and Discussion

Total Soluble Protein Concentration and Yield

Protein extraction typically starts with breaking the protective cell wall and plasma membrane. As such, there is a definite release of other intracellular components that interfere with subsequent proteomic analyses, such as polyphenols, polysaccharides, proteases, lipids, and numerous secondary metabolites. Obviously, these components have to be removed from the protein sample. Therefore, we applied the washing step with ice-cold acetone in this study to effectively remove the polyphenol and pigment. This resulted in acetone-dried powder (AcDP) followed by the protein extraction process. The phenol extraction method was used in this study due to excellent results reported by Carpentier et al. (2005), who compared several extraction protocols and found that phenol extraction was the most powerful. Saravanan and Rose (2004) also proved that phenol extraction is preferable when dealing with recalcitrant plant tissue.

The results showed that *C. lentillifera* had a total soluble protein concentration of 4.84 ± 0.22 µg/uL and a protein yield of 1403.65 ± 15.61 µg/g AcDP. The protein content of marine algae varies to a large extent depending on species and season. In general, the highest levels of protein are found in the red species (maximum 47%(w/w) dry weight), with moderate to low levels found in green (9–26%(w/w) dry weight) and brown (3–15%(w/w) dry weight) phyla (Fleurence, 2004). In some red seaweed, protein levels can be as high as 35%(w/w) (*Palmaria palmata* (dulse)) and 47%(w/w) (*Porphyra tenera* (nori)), while the green alga *Ulva pertusa* (anori) can contain up to 26%(w/w) protein (Fleurence, 2004; Wong & Cheung, 2000).

Mass Spectrometry Analysis and Database Searches

The tandem-mass spectra were analyzed by PEAKS studio 7 *de novo* sequencing software to generate amino acid sequences. The independence of a sequence database makes *de novo* sequencing the preferred method for identifying novel peptides and studying unsequenced organisms. Even when the peptide sequence is in a database, *de novo* sequencing can significantly help improve the database-search-based peptide identification since the match between the de novo sequence and the database sequence confirms the correctness of the identification (Zhang et al., 2012). The results showed that 76 peptides were mapped to the selected protein, and all peptides ranged from 6 to 15 amino acid residues (Table 1). Meanwhile, 145 were *de novo* peptide sequences, and all these peptides range from 5 to 12 amino acid residues (Table 2).

Potential Biological Activity Profile

Bioinformatics has become a powerful tool for peptide research (Minkiewicz et al., 2008), including *in silico* prediction of the release of bioactive peptides from food proteins (Vercruysse et al., 2009). The BIOPEP-UWM database is a bioinformatics tool enabling the detection of biologically active fragments in protein sequences, the classification of proteins as potential sources of bioactive fragments, to simulate protein hydrolysis and find peptides that can be released by a given enzyme or, as a result, of the combined action of two or three enzymes (Minkiewicz et al., 2008).

A successful prediction of the activity of protein hydrolysates using the BIOPEP-UWM database and its search engine has been recently described by Cheung et al. (2009) on the example of angiotensin I-converting enzyme inhibitory activity of oat protein hydrolysates. Data concerning angiotensin I-converting enzyme inhibitory peptide with the LQP sequence have also validated the reliability of computational predictions using the BIOPEP database. This peptide has been discovered by Miyoshi et al. (1991) in a-zein hydrolysates. It has been found in the profiles of potential biological activity of bovine b-casein and wheat a/b-gliadin, obtained in silico, published by Dziuba et al. (1999). The above peptide has been detected experimentally in cheese (Tonouchi et al., 2008) and wheat grain milling by-products (Nogata et al., 2009). Physiologically active peptides form a complex and highly diversified group of compounds with regard to their terminology, structure, and functions. Many biologically active peptides are multifunctional, performing regulatory functions and directly affecting various developmental and metabolic processes.

Table 1. List of the matched peptide sequence identified by nLC-MS/MS-Orbitrap in the protein extract of C. lentillifera after digestion with Trypsin. Spectra analysis was performed using the PEAKS Studio 7 Software using the search parameters described in the materials and methods section.

No.	Peptide	No.	Peptide	No.	Peptide
1	LSGGDHLHSGTVVGK	27	AGIMLSPTFVK	53	IPYDQQIK
2	LSGGDHIHSGTVVGK	28	DNFVEKDR	54	LYSIASSR
3	HYAHVDC(+57.02)PGHADYVK	29	VAINGFGR	55	AVSLVLPKLK
4	AQLGEIFEFDR	30	AVVISVIDNLVK	56	PDTFAELK
5	SLLGC(+57.02)TIKPK	31	IGINGFGR	57	TEDC(+57.02)VGC(+57.02)KR
6	VINTWADIINR	32	LGINGFGR	58	APGFGDR
7	VLNTWADIINR	33	ELEVIHAR	59	IAAFDGER
8	VINTWADILNR	34	EIEVIHAR	60	SVDETLR
9	DHGLLLHIHR	35	VYLGPETTR	61	SAPLGGTSGQSAAAGLR
10	LEDLRIPPAYAK	36	IQPDEISSIIR	62	APGFGDRR
11	IFGVTTLDVVR	37	ASQIASAPR	63	YGSLLR
12	LFGVTTLDVVR	38	HLPGFIEK	64	VLGFSLR
13	EVTLGFVDLMR	39	VAEYTLK	65	EAHTHIK
14	NKITITNDKGR	40	ALRLEDLR	66	GLFIIDK
15	VPLILGVWGGK	41	GNAPGAAANR	67	AVYEC(+57.02)LR
16	VHTVVLNDPGR	42	GHRQELTR	68	VVDLLAPYRR
17	VHTVVINDPGR	43	AWMAAQDQPHEK	69	AGNHEAVVK
18	AGFAGDDAPR	44	GGLDFTK	70	TAGGGGAAAVR
19	TFQGPPHGIQVER	45	LINLSGK	71	ELIIGDR
20	YRELEVIHAR	46	LGC(+57.02)TIKPK	72	IGPLGLSPK
21	YREIEVIHAR	47	RDHVLYGK	73	LGPLGLSPK
22	DVMASESAAFR	48	GSTFLDPK	74	WAKPGHFSR
23	DGVYPEKVNAGR	49	YNKKPTLTSR	75	RC(+57.02)LVCPGEQPK
24	AMHAVIDR	50	GKLNGIALR	76	NQFYVTPK
25	AMHAVLDR	51	GKLNGLAIR		
26	ESTLHLVLR	52	GKLNGLALR		

The results of evaluating peptide sequence (protein peptide) as the precursor of bioactive peptide for the protein peptide of *C. lentillifera* using BIOPEP-UWM database are shown in Figure 1. A total of 159 fragments (motifs) of peptide sequences have been detected from 76 protein peptides. Overall, they showed only 13 biological activity characteristics of bioactive peptides. The majority of matches consisted related to motifs with a potential of ACE inhibitory activity of 97 different motifs (60.00%), followed by inhibitor having 16 different motifs (11.00%), antioxidative having 18 different motifs (11.00%), stimulating having 9 different motifs (6.00%).

Meanwhile, regulating has 4 different motifs (3.00%), antiamnestic has 3 different motifs (3.00%), and antithrombotic has 2 different motifs (3.00%). The other six, neuropeptide, hypotensive, immunomodulating, opioid, bacterial permease ligand, and activating ubiquitin-mediated proteolysis, generally only 1.00% and less. The motif sequence of LG in the group of ACE inhibitors showed the most frequent (12 times).

Table 2. List of the *de novo* peptide sequence identified by nLC-MS/MS-Orbitrap in the protein extract of *C. lentillifera* after digestion with Trypsin. Spectra analysis was performed using the PEAKS Studio 7 Software using the search parameters described in the materials and methods section

No.	*de novo* Peptide	No.	*de novo* Peptide	No.	*de novo* Peptide
1	LAC(+57.02)EAC(+57.02)LQAR	50	FGPNLR	98	HVVFGLVK
2	SGPEDKFR	51	LC(+57.02)YLK	99	TKLC(+57.02)YLK
3	LEDLR	52	GYLSYHDGR	100	LSMENQR
4	SGPEDKFR	53	LLFPEEVLPR	101	LTFTGSNPR
5	LLGVTTLDVVR	54	LVNNTYAK	102	LLFPEEVLPR
6	LEDLR	55	SAAHC(+57.02)YK	103	SKPSTLSLR
7	YELLTR	56	LSYHDGR	104	YLVENQK
8	LALQM(+15.99)C(+57.02)AKK	57	NVYTGLFYGR	105	VC(+57.02)NYVSWLK
9	YELLTR	58	LC(+57.02)YLK	106	FLGLNQLGEK
10	LGMDEELLR	59	LENNFR	107	M(+15.99)LADAMKK
11	VGGAFLQR	60	VSGTC(+57.02)VGSYR	108	GYLSYHDGR
12	LLGVTTLDVVR	61	VLTDDGVALR	109	TDHDHC(+57.02)WC(+57.02)K
13	VGGAFLQR	62	HGEFSK	110	LLTTVAGFDR
14	LGMDEELLR	63	LSELVTDWHR	111	LGENSDPVSVK
15	VLGSVTVR	64	LSELVTDWHR	112	VCNYVSWLK
16	HGMHFR	65	LEFYGPNR	113	LAELLR
17	VAEYTLR	66	WFTSELESNR	114	LGHHNEHLK
18	DTLADLHAK	67	HGEFSK	115	TDHDHC(+57.02)WC(+57.02)K
19	VAEYTLR	68	LEFYGPNR	116	ASGDVTLR
20	FVLNR	69	LEFYGPNR	117	DFTC(+57.02)DTSR
21	HYTEALKR	70	ASGLGVGNAAR	118	M(+15.99)DALTKK
22	LTEC(+57.02)LER	71	WSTDGGLFLR	119	LHGMHFR
23	AVASSQSTFR	72	M(+15.99)DALTKK	120	AFKPLHDR
24	LEVGFR	73	YAC(+57.02)TVGSR	121	MEGTTVNAR
25	LLYVEK	74	YTDLLLR	122	EAGEAFAVSFAR
26	LPAQALK	75	ELLGTLR	123	MNFLLK
27	LELGTLR	76	FLADAMQK	124	YYGALFPFYVNK
28	STAQTAAR	77	TSLGHLESLR	125	VEFGLTNSVR
29	LKSAASLNSR	78	VGSDSELLEAFK	126	MHFAVR
30	VLETDLLAAFR	79	LPAQALK	127	LLVAGATGR
31	STAQTAAR	80	VLTDDGVALR	128	YVLASLGSLSK
32	YLNSEFR	81	NLYWATGVR	129	KYQMK
33	YETDFGLFPR	82	SGFLLER	130	TPLHLAALK
34	VLETDLLAAFR	83	SKPSTLSLR	131	NSLTGVTPSR
35	YETDFGLFPR	84	VSLSTC(+57.02)R	132	KYQMK
36	YTELVK	85	ESLVSYNPFK	133	LLVAGATGR
37	STAQTAAR	86	LSTALDSR	134	LTFTGSNPR
38	STAQTAAR	87	EAGEAFAVSFAR	135	VLLGHSER
39	LEANFR	88	LAGFTFGPDR	136	YLSMAK
40	TKLC(+57.02)YLK	89	VMHFAVR	137	QVLTLHK
41	LEANFR	90	LELPNEVSR	138	QYKMK
42	YTELVK	91	FGPNLR	139	HLDLSAVR
43	LALQQVR	92	GLHLGEQR	140	LGHHNEHLK
44	RFEFYAK	93	QEFSSC(+57.02)LLR	141	LEFYGPNR
45	YLNSEFR	94	SVKMEVR	142	VVHKTM(+15.99)GLK
46	WSTDGGLFLR	95	TFDLAK	143	LALQQVR
47	MHFAVR	96	TDPEFLRK	144	M(+15.99)LGLNKLGEK
48	MHAVLDR	97	SPLQVR	145	AKTVPLSAR
49	LSTALDSR				

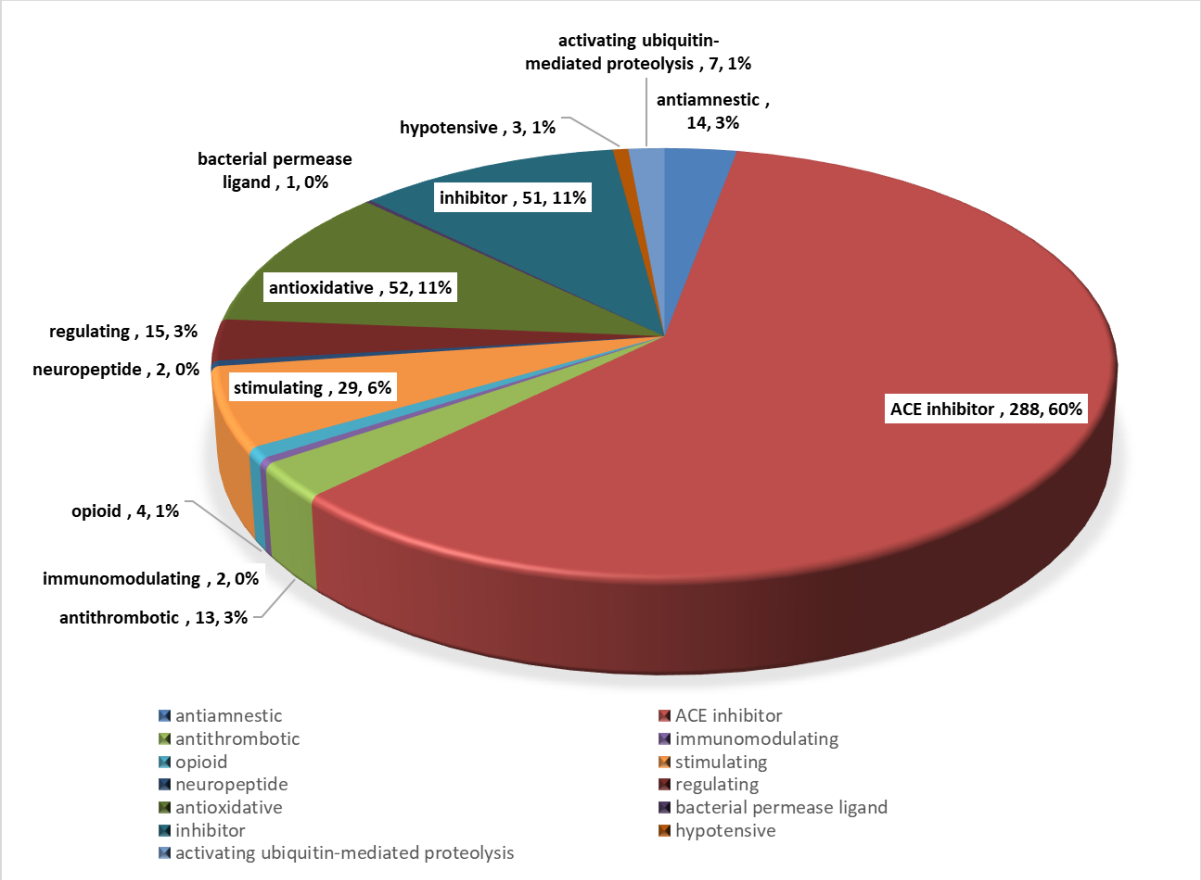

Figure 1. Distribution of bioactive peptides/motifs from *C. lentillifera* of protein-peptide according to their main activity (presented as a total number of motifs occurring and percentage).

The results of evaluating peptide sequence (*de novo* peptide) as the precursor of bioactive peptide for the protein peptide of *C. lentillifera* using BIOPEP-UWM database are shown in Figure 2. A total of 159 fragments (motifs) of peptide sequences have been detected from 145 *de novo* peptides. Overall, they showed 15 biological activity characteristics of bioactive peptides. The majority of matches consisted related to motifs with a potential of ACE inhibitory activity of 78 different motifs (55.00%), followed by inhibitor having 14 different motifs (14.00%), antioxidative having 19 different motifs (9.00%), stimulating having 7 different motifs (8.00%), regulating has 3 different motifs (2.00%), hypotensive has 2 different motifs (2.00%), antithrombotic has 2 different motifs (2.00%), neuropeptide has 2 different motifs (2.00%), and activating ubiquitin-mediated proteolysis has 2 different motifs (2.00%), The other six, antiamnestic, neuropeptide, immunomodulating, opioid, bacterial permease ligand, and antibacterial, generally only 1.00% and less. The motif sequence of LL in the group of stimulating and inhibitors showed the most frequent (23 times).

The results of computer-aided prediction of the bioactive peptide release from plant proteins indicate a relatively good possibility of obtaining biologically active peptides. The location of bioactive fragments in the hydrophilic part of a plant protein molecule can be an essential aspect in the production of functional food, i.e., food with special desired and designed features, as well as in the production of Nutraceuticals (Darewicz and Dziuba, 2000). Knowledge about the structure of bio-macro molecules can help investigate their structure-function relationship. Bioinformatic methods applied in biotechnology or biochemistry have become increasingly popular due to the short time required to obtain results, low research costs, the possibility of recording results in text files, and their good reproducibility. At the same time, as computer science develops and methods are improved.

In view of the Figure 2, a computational analysis may be successfully used for rapid screening of protein sequences to predict the potential biological activity of selected fragments and to find a way to liberate them. As a result, seaweed sample *C. lentillifera* has great potential to supply functionally significant peptides. The findings of the study can be exploited in the development of foods with special health claims (e.g. treatment of hypertension) as well as in identifying new applications in food. In the future, seaweed-derived bioactive

peptides may be important components in foods sustaining health and the prevention of diseases such as cardiovascular diseases.

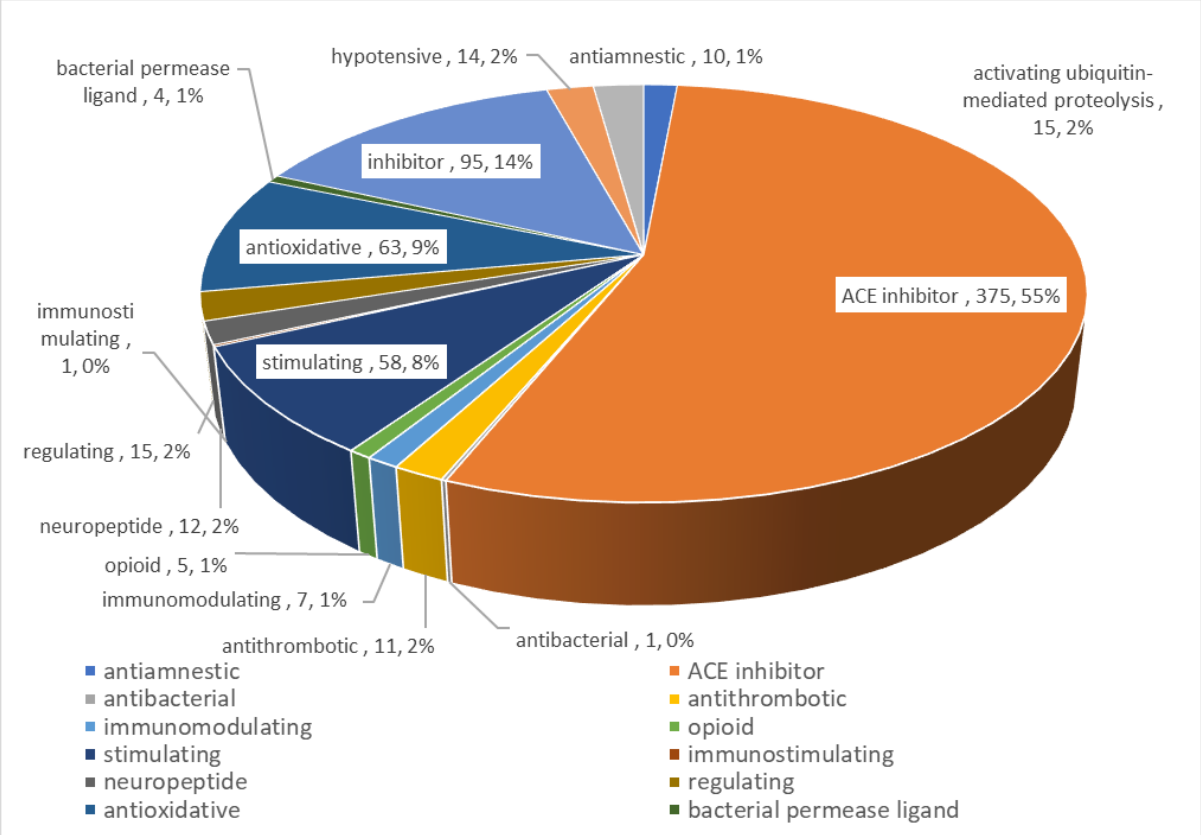

Figure 2. Distribution of bioactive peptides/motifs from *C. lentillifera* of *de novo* peptide according to their main activity (presented as a total number of motifs occurring and percentage).

Conclusion

In conclusion, this *in silico* study shows that this protein raw material (*C. Lentillifera*), which remains a relatively untapped reservoir, can act as a resource for generating bioactive peptides with potential health-promoting and disease-preventing properties. In general, various bioactive peptides can be found in these seaweed protein extracts, and the studies showed that these seaweed samples contain protein sequences that can be a potential source of ACE and inhibitory (dipeptidyl peptidase IV inhibitory) activity bioactive peptides. The study's findings can be exploited in the development of foods with special health claims (e.g. treatment of hypertension) as well as in identifying new applications in food. These investigations may significantly contribute as an initial step that can improve market value for seaweed produce and provide new insights into human nutrition.

Recommendations

Further studies are still required to better understand the possible adverse effects exerted by the respective peptides or their by-products, which would inevitably be contained in such foods. Safety requirements include the absence of toxicity, cytotoxicity, and allergenicity.

Scientific Ethics Declaration

The authors declare that the scientific, ethical, and legal responsibility of this article published in EPHELS journal belongs to the authors.

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