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ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF SMALL PEPTIDES ISOLATED FROM MARINE ALGAE BY GREEN METHODS

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Abstract

This study aimed to investigate new green methods based on combined ultrasound- and enzyme-assisted technologies to isolate bioactive peptides from Cladophora vagabunda green macroalga. Also, their antioxidant and antiproliferative activity was analysed in view of biomedical applications. Algal powder was sonicated in alkaline buffer, pH 8, at 37 °C, for 1 h. The extract was treated with proteinase K and alcalase, respectively, to obtain two protein hydrolysates. Each hydrolysate was fractionated by centrifugal ultrafiltration using filter membranes with molecular weight cut-off (MWCO) at 3 kDa, to separate small peptides. The fractions were analyzed for Trolox Equivalent Antioxidant Capacity (TEAC). The results showed higher capacity of hydrolysates obtained by alcalase treatment to scavenge free radicals. Additionally, the antiproliferative activity was evaluated in HT-29 tumor cells cultivated in the presence of peptides obtained by alcalase treatment and the results showed a decrease of cell viability below 70% after 48 h of cultivation. In conclusion, peptide fractions isolated by green techniques from C. vagabunda alga had significant biological activity and are recommended for further testing as therapeutic agents.

Key words*: algal peptides, Cladophora vagabunda, enzymatic hydrolysis, marine bioactive compounds, sonication.*

INTRODUCTION

High quantities of animal proteins are found in sources such as meat, eggs and milk, while vegetable proteins are found in soy and maize. Lately, the micro- and macroalgal biomass was considered a viable alternative source of proteins (Espinosa-Ramirez et al., 2023). The protein production from marine macrophyte algae (2.5-7.5 tons/ha/year) is higher than that from terrestrial cultures of soy (0.6-1.2 tons/ha/year) (O'Connor et al., 2022). Additionally, proteins from edible seaweeds are nutritionally important, containing high quantities of essential amino acids like lysine, tryptophan, methionine, valine, thus being recommended for human diet (Kazir et al., 2019). The protein extraction yield represented up to 47% when using red algae species (Rhodophyceae) and up to 29% in green (Chlorophyceae) and brown (Phaeophyceae) algae species, the quantity varying according to geographical area and harvesting season (Vieira et al., 2018). However, traditional technologies for algal protein extraction are time consuming and economically unviable (Bleakley & Hayes, 2017).

Currently developed technologies for bioactive compounds extraction from algal biomass are based on green methods of ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and enzyme-assisted extraction (EAE). In case of UAE, the effect of acoustic cavitation could improve seaweed proteins bioavailability by formation and growth of vapor bubbles and facilitating penetration of solvent into the cells to release intracellular compounds (Arya et al., 2023). An ultrasound-assisted treatment in acid or alkaline conditions was efficient for protein extraction from *Ascophyllum nodosum* brown alga (Pan-utai et al., 2023). On the other side, MAE takes place by transfer of microwaves energy to polar water molecules, increasing their vibration, collision, and intracellular heating, and thus leading to an effect of pressurization and algal cell membrane damage (Grosso et al., 2015). EAE using enzymes (cellulases, agarases, xylanase, carragenase) for digestion of cellulose-type polysaccharides (galactans, xylans, fucoidans, laminarin, alginates, carrageenan) present in the rigid cell wall of algal cells were useful in protein extraction from red alga species

Chondrus crispus, *Gracilaria verrucosa* and *Palmaria palmata* (Fleurence et al., 1995).
Green algae present a chemical composition rich in polysaccharides, phenolic compounds, proteins and amino acids, fatty acids, vitamins and minerals (Lafarga et al., 2020). Extracts of various green algae demonstrated immonomodulatory, antimicrobial, anti-inflammatory and antitumoral activity (Surayot et al., 2016). The genus Cladophora is comprising marine green macrophyte algae with filamentous branched multinucleate cells, widespread from temperate to arctic and tropical waters (Michalak & Messyasz, 2020). *Cladophora vagabunda* is abundantly found in the Black Sea, it has an invasive tendency, but it is still an underexploited biological resource (Marin & Timofte, 2011). Cladophora biomass was reported as valuable raw material for agriculture and cosmetics (Messyasz et al., 2015) and extraction of volatile compounds and fatty acids from *C. vagabunda* indicated applications as bioadditives and biopreservatives (Horincar et al., 2014). In this context, the present paper aimed to investigate new green methods for the isolation of bioactive peptides from *C. vagabunda* macroalga and their antioxidant and antiproliferative activity, in view of valorization as natural ingredients with biomedical applications.

MATERIALS AND METHODS

Biological material and chemical reagents

The biological material consisted of *C. vagabunda* green macrophyte alga collected from the Black Sea between 2 Mai and Vama Veche, in August 2022. The algae were extensively washed in cold tap water to remove the sand, dried in an oven (Memmert, Germany) at 30 °C and ground using an electric grinder until a fine green powder was obtained.
All chemical reagents used in this study were of analytical grade. 2,4,6-trinitrobenzene sulfonic acid (TNBS), 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), proteinase K (E.C. 3.4.21.64), alcalase (E.C. 3.4.21.14), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other chemicals were purchased from Sigma-Aldrich (Germany), unless otherwise stated. Human HT-29 epithelial cell line derived from colorectal adenocarcinoma, Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS) and penicillin-streptomycin-neomycin antibiotic mixture (PSN) were purchased from Sigma-Aldrich (Germany).

Extraction of algal proteins based on ultrasound-assisted technology

The extraction of proteins from *C. vagabunda* powder was carried out in TES buffer 0.05 M, pH 8 (1:20, m/v) by ultrasonication in a sonication bath (Elma, Germany), at 60 kHz, at a temperature of 37 °C, for 1 h (Figure 1). The temperature was controlled throughout the process not to exceed 37 °C, by sequencing the ultrasonication process in 5 on-off cycles, each lasting 10 min with a break of 2 min. Then, the process continued by magnetic stirring, at 4 °C, for 24 h. The extraction was repeated twice using the residue obtained after centrifugation of the solution at 9000 g, at 4 °C, for 20 min, and all supernatants were pooled together (P1).
The extraction yield was determined on a dry weight (d.w.) basis using the following formula:

$$\text{extraction yield (\%)} = \text{d.w.}_{\text{final}} / \text{d.w.}_{\text{initial}} \times 100$$

The protein content was determined by BCA assay, as previously described (Mihai et al., 2021).

Preparation and purification of algal peptides by enzyme-assisted technology

Enzymatic hydrolysis of protein extract (P1) was carried out using specific neutral proteases of microbial origin under specific conditions of pH and temperature. Thus, the algal extract aliquots were treated with 3% proteinase K in 0.05 M Tris buffer, pH=8, supplemented with 1 mM $CaCl_2$ (PP1) and 3% alcalase in 0.05 M Tris buffer, pH=8 (PA1), respectively (Figure 1). The mixtures were incubated in a shaking water bath (Witeg, Germany), at 55 °C, for 3 h. The pH of the reaction mixture was periodically verified throughout the enzymatic process and maintained at the initial optimal value. At the end of the incubation period, the mixtures were heated at 95 °C for 5 min, to inactivate the enzyme. Then, the solutions were cooled at room temperature and centrifuged at 9000 g, at 4 °C, for 20 min. Each hydrolysate was subjected to centrifugal ultrafiltration using cellulose membrane filter units with MWCO at

3 kDa (Amicon, Germany) centrifuged at 7500 g in successive cycles of 30 min, according to manufacturer's instructions. Thus, two fractions were separated from each hydrolysate, the permeates (PP1 and PA1 fractions with MW<3 kDa) and the retentates (PP1 and PA1 fractions with MW>3 kDa).

Determination of the degree of hydrolysis

The degree of hydrolysis was determined as free amino groups quantified by TNBS assay, as previously described (Craciunescu et al., 2011). Thus, samples of hydrolysates (PP1, PA1) and the non-hydrolysed protein extract (P1) (0.25 ml) were mixed with 0.05% TNBS in 0.05 M TES buffer, pH=8 (1 ml) and incubated in a shaking water bath (Witeg, Germany) at 50 °C, in the dark, for 1 h. Then, 0.1 N HCl (3 mL) was added and the incubation continued in the water bath, at 50 °C, in the dark, for 10 min. After cooling at room temperature, distilled water (5 mL) was added. The absorbance (Abs) was read in quartz cuvettes at a wavelength of 346 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). Total hydrolyzate was prepared by P1 incubation in 6 N HCl, at 110 °C, 24 h. The degree of hydrolysis was calculated using the following formula:

$$\text{degree of hydrolysis (\%)} = (Abs_{hydrolysate} - Abs_{non\text{-}hydrolysed}) / (Abs_{total\ hydrolysate} - Abs_{non\text{-}hydrolysed}) \times 100$$

Determination of antioxidant capacity

The antioxidant capacity was determined by TEAC assay, as previously described (Gaspar-Pintiliescu et al., 2022). A stock solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1, v/v) and incubation at room temperature, in the dark, for 16 h, to allow free ABTS radicals formation. For the experiment, the stock solution was diluted with distilled water to give an Abs of 0.70 ± 0.02 at a wavelength of 734 nm (blank). The samples (100 µL) were mixed with ABTS reagent (1 mL) and incubated at room temperature, in the dark, for 10 min. The Abs of the reaction mixtures was read at 734 nm using a V650 UV-VIS spectrophotometer (Jasco, Japan). A standard curve was built using Trolox, a synthetic antioxidant, in the range of concentrations of 10-150 µM. The antioxidant capacity was calculated using the following formula:

$$TEAC = (Abs_{blank} - Abs_{sample}) / Abs_{blank} \times 100$$

The results were expressed as mM Trolox equivalents (TE) per g d.w.

Determination of antiproliferative activity

Cell culture and treatment

Human tumor HT-29 epithelial cells were seeded in the wells of a 96-well culture plate at a density of $5x10^4$ cells/mL and cultured in DMEM medium supplemented with 10% FCS, 1% glutamine, and 1% PSN antibiotic mixture. The plates were incubated in standard conditions, at a temperature of 37 °C, in 5% CO_2 atmosphere, for 24 h, to allow cell adhesion. Then, the culture medium was replaced with fresh culture medium supplemented with FCS containing different concentrations of algal peptides in the concentration range of 0.16-2.5 mg/mL. The plates were incubated in standard conditions for 24 and 48 h, respectively. The cells cultivated in MEM medium without treatment, in standard conditions served as negative control.

MTT assay

Cell proliferation was evaluated by MTT assay based on the reduction of yellow tetrazolium salts to purple insoluble formazan by succinate mitochondrial dehydrogenases in metabolically viable cells, a reaction mediated by the reduction NADH to NADPH (Oprita et al., 2008). Thus, at the end of the incubation period, the culture medium in each well was replaced with 0.25 mg/mL MTT solution (100 µL) and the plates were incubated at 37 °C, in 5% CO_2 atmosphere, for 3 h. Then, the MTT solution was replaced with isopropanol (100 µL) to solubilize the formazan crystals by gentle stirring, on a horizontal shaker, for 15 min. The Abs was read at a wavelength of 570 nm using a SpectroStar Nano microplate reader (BMG Labtech, Germany). The Abs values were directly proportional to the number of viable cells. Cell proliferation was calculated using the following formula:

$$\text{cell proliferation (\%)} = Abs_{sample} / Abs_{control} \times 100$$

Statistical analysis

The experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD) (n=3). Statistical analysis was performed by two-tailed, two-sample equal variance Student *t*-test on control-sample pairs of interest. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSIONS

In the present study, an experimental protocol based on combined green technologies of UAE and EAE was optimized at laboratory level for sustainable valorization of *C. vagabunda* green macrophyte algal biomass to obtain peptide fractions with bioactivity towards free radicals scavenging and antiproliferative activity in tumor cell culture, in order to be used as natural therapeutic agents (Figure 1).

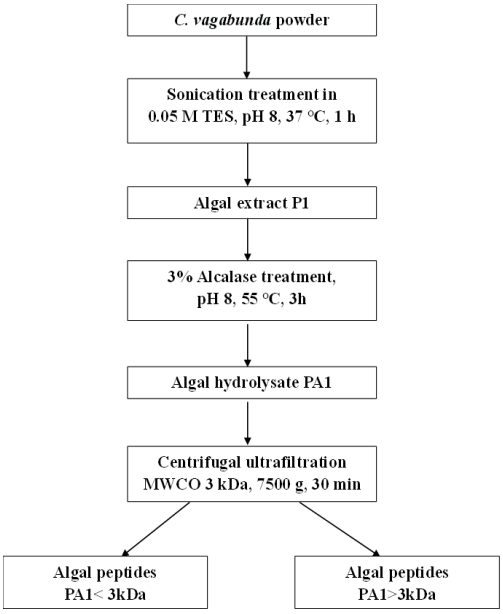

Figure 1. Scheme of combined UAE and EAE technologies to isolate algal bioactive peptides under controlled conditions from *C. vagabunda*

Preparation of algal proteins by UAE

First, an algal protein extract was obtained from *C. vagabunda* green alga powder by ultrasonication with a yield of 23.68%, calculated on a d.w. basis. This value was similar to data reported in previous studies on different green algae from *Ulva* species (7-29%) (Pimentel et al., 2019).

This high extraction rate confirmed the efficacy of the sonication-based optimized technology. It is known that macrophyte algae are characterized by the presence of polysaccharidic cell walls that play an important role in mechanical shear resistance (Shao & Duan, 2022). As a result, in the present study, the extraction of proteins from *C. vagabunda* green macroalga was conducted by ultrasonication treatment at low frequency and high intensity (20-100 kHz), which allowed the destruction of cell walls for improving the bioactive compounds extraction yield. Additionally, the ultrasound-assisted technology applied for a short processing period allowed extraction of thermolabile proteins without damaging their structure and properties, in accordance to previous reports (Ojha et al., 2020; Prandi et al., 2022). The alkaline buffer (pH=8) improved the solubilization of large peptides and proteins from cell cytoplasm and break down of their globular structure, but also facilitated the easy release of small peptides and free amino acids from algal biomass. Similar studies reported that the yield of protein extraction from *Nannochloropsis* spp. was doubled in alkaline solvents (pH=8), compared to water (O'Connor et al., 2022; Parniakov et al., 2015).

Preparation of algal peptides by EAE

Algal peptides were obtained using specific neutral proteases (3% proteinase K and 3% alcalase solutions) with broad action and stability over large pH and temperature domains, to ensure controlled degradation (Figure 1). An indicator of protein degradation and peptides formation is the number of free amino groups, determined in the present study by TNBS assay. The degree of enzymatic hydrolysis of algal proteins reached values of 9.22% under proteinase K treatment (PP1) and 45.87% under alcalase treatment (PA1). These data indicated an efficient hydrolysis of *C. vagabunda* protein extract by alcalase under mild optimal reaction conditions (pH 8, 55 °C, 3 h).

Each hydrolysate was subjected to centrifugal ultrafiltration through membranes with MWCO of 3 kDa, which allowed separation of 2 fractions (permeates with MW<3 kDa and retentates with MW>3 kDa). The results of

protein content analysis in permeates and retentates are given in Table 1.

Table 1. Protein content of protein hydrolysate and peptide fractions from *C. vagabunda*

Protein content (mg/g d.w.)	
Proteinase K hydrolysate	
PP1 (total hydrolysate)	54.52 ± 2.74
PP1 < 3 kDa (permeate)	20.10 ± 1.22
PP1 > 3 kDa (retentate)	106.17 ± 7.91
Alcalase hydrolysate	
PA1 (total hydrolysate)	73.91 ± 3.35
PA1 < 3kDa (permeate)	44.52 ± 2.42
PA1 > 3kDa (retentate)	182.49 ± 7.63

The results represent mean ± SD (n=3).

The results showed that alcalase hydrolysate (PA1) had higher protein content (73.91 mg/g d.w.), compared to proteinase K hydrolysate (PP1) (54.52 mg/g d.w.). Also, peptide fractions isolated from alcalase hydrolysate had ~2-fold higher protein content than those isolated from proteinase K hydrolysate (Table 1). All these data allowed selection of alcalase as optimal protease for preparation of algal peptides from *C. vagabunda* and obtained fractions were further investigated for bioactivity *in vitro*.

Antioxidant capacity of *C. vagabunda* peptide fractions

The high protein content of macrophyte algae and their antioxidant activity is an important reason for separation and valorization in biomedical applications (Surayot et al., 2016).

The antioxidant capacity of peptide fractions isolated from *C. vagabunda* green alga by UAE and alcalase treatment was determined by TEAC assay. The results are presented in Table 2.

Table 2. Antioxidant capacity of alcalase hydrolysate and peptide fractions from *C. vagabunda*

Sample	TEAC (mM TE/g d.w.)
PA1 (total hydrolysate)	163.78 ± 7.34
PA1<3 kDa (permeate)	123.67 ± 5.91
PA1>3 kDa (retentate)	139.43 ± 4.25

The results represent mean ± SD (n=3).

The results showed that all peptide samples presented high potential to scavenge free ABTS radicals. It was observed a decrease of the antioxidant capacity of peptides from permeate fraction, compared to alcalase hydrolysate and retentate. This could be due to smaller size of peptides from permeate containing more amino and carboxyl terminal groups, which decreased the antioxidant activity, as previously reported (Zhao et al., 2020).

The antioxidant activity of peptides could be correlated to their MW, but also to the hydrophobic nature of the amino acids present in their sequences. Thus, in the case of VECYGPRPQF peptide isolated from *Chlorella vulgaris* green macrophyte alga, rich in hydrophobic and aromatic amino acids, it was reported a potent antioxidant activity (Sheih et al., 2010). In the present study, the use of alcalase for algal protein hydrolysis could lead to hydrophobic peptides with increased antioxidant activity due to its known activity of preferential cleavage of the protein before aromatic amino acids, compared to proteinase K hydrolysis that had broader specificity and also cleaved before aliphatic residues and glycine residues.

Previous studies on *Palmaria palmata* macrophyte alga identified mycosporine-like amino acids, such as palythine, porphyra-334, shinorine-330, asterin, palythinol, palythene, usujirene, produced as low MW water-soluble secondary metabolites (Yuan et al., 2009). Their structural characteristics consisting of a cyclohexanone or hexenimine core conjugated to the nitrogen of an amino acid allowed significant antioxidant activity (Bedoux et al., 2014). Similar molecules were isolated from *Porphyra umbilicalis* macrophyte alga and used to obtain Helioguard 365® sunscreen, exhibiting antioxidant activity, capacity to absorb UV radiation and dissipate energy in the form of heat (Pandey et al., 2017), limiting the negative effect of fotons on cellular components (Pimentel et al., 2019).

Antiproliferative activity of *C. vagabunda* peptide fractions

The antiproliferative activity of peptide fractions isolated from *C. vagabunda* green alga was assessed in human tumor HT-29 epithelial cells by MTT assay. The results are presented in Figure 2.

The results showed that alcalase-treated peptide fraction PA1<3 kDa presented similar Abs values at 24 h of cultivation and significantly

($p<0.05$) higher Abs values at 48 h of cultivation, compared to the control, indicating stimulation of cell proliferation. The algal hydrolysate PA1 and peptide fraction PA1>3 kDa could significantly ($p<0.05$) inhibit tumor cell proliferation after 48 h of cultivation, at all tested concentrations, compared to the control. The highest activity was 2-fold inhibition of cell proliferation at 2.5 mg/mL PA1>3 kDa, after 48 h of cultivation. *In vitro* data demonstrated the antiproliferative activity of algal bioactive peptides isolated from *C. vagabunda* in the experimental model based on human HT-29 colon epithelial cells. These *in vitro* results correlated direct proportional to the antioxidant activity of algal peptides and their MW.

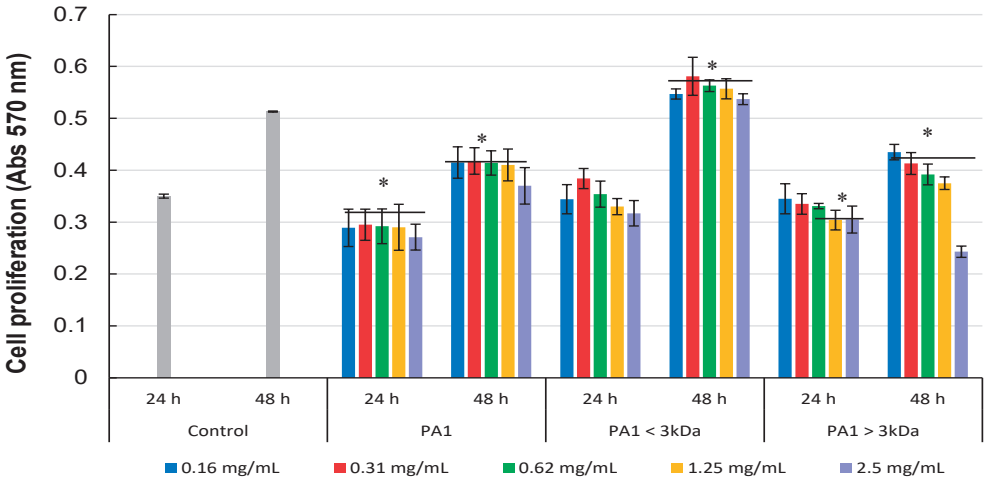

Figure 2. Antiproliferative activity of alcalase hydrolysate (PA1) and peptide fractions from *C. vagabunda* green alga. The results represent mean ± SD (n=3). *$p<0.05$, compared to control.

Previous studies have reported that two peptides VPGTPKNLDSPR and MPAPSCALPRSVVPPR identified in trypsin hydrolysate of *Porphyra haitanesis* protein extract revealed inhibition of human MCF-7 breast cancer cells and human HepG-2 liver cancer cells proliferation and cell cycle arrest inducing cell apoptosis (Fan et al., 2017). The study on papain hydrolysate of *Pyropia haitanensis* protein extract showed antiproliferation effect of QTDDNHSNVLWAGFSR peptide on human HepG-2 liver cancer cells at a concentration of 500 µg/mL that induced low inhibition of human normal liver cells (Mao et al., 2017).

CONCLUSIONS

Green biotechnologies based on combined UAE and EAE using specific neutral proteases (proteinase K, alcalase) were established to extract bioactive peptides from *C. vagabunda* green macroalga. Alcalase was selected as optimal enzyme for the isolation of peptide fractions from *C. vagabunda* green macroalga due to higher degree of hydrolysis, compared to proteinase K. The hydrolysate obtained by algal protein digestion using alcalase and peptide fractions isolated after centrifugal ultrafiltration showed significant antioxidant capacity as free ABTS radicals scavenging potential. The experimental model *in vitro* demonstrated that the isolated bioactive peptides could inhibit cell proliferation in human tumor HT-29 epithelial cells.

In conclusion, the algal peptides isolated from *C. vagabunda* had significant biological activity and are recommended for further testing as therapeutic agents.

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