Antioxidant activity and techno-functional properties of protein extracts from *Caulerpa prolifera*: Optimization of enzyme-assisted extraction by response surface methodology

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Summary

In this study, ultrasound-assisted enzymatic extraction was performed to extract proteins from *Caulerpa prolifera* and the techno-functional properties of the protein extracts were investigated for the first time. The effect of extraction temperature (30–40 °C), extraction time (60–120 min) and substrate/enzyme (S/E) ratio (5–15) on protein content (*PC*), total phenolics content (*TPC*) and antioxidant activity (*AOA*) was investigated using response surface methodology (RSM). According to the results, optimum conditions were determined as extraction temperature of 30 °C, extraction time of 60.03 min and S/E of 5. The values of *PC*, *TPC*, AOA_{CUPRAC} (AOA determined by cupric reducing antioxidant capacity assay) and AOA_{DPPH} (AOA determined by 2,2-diphenyl-1-picrylhydrazyl assay) of the extracts obtained under optimum conditions were 51.57 g·kg^{-1} dry weight (dw), 31.12 g·kg^{-1} dw (expressed as gallic acid equivalents), 4.45 g·kg^{-1} dw and 0.83 g·kg^{-1} dw (expressed as Trolox equivalents), respectively. Water and oil absorption capacity, emulsion activity-stability and foaming capacity-stability of the proteins extracted under optimum conditions were determined as 54.0 ± 5.0 %, 210.0 ± 15.0 %, 41.4 ± 2.3 %, 41.7 ± 0.1 %, 26.5 ± 1.5 % and 26.7 ± 2.4 %, respectively. These research findings indicate that *C. prolifera* proteins, as an alternative protein source, offer promising opportunities for functional food applications.

Keywords

Caulerpa prolifera; macroalgae; protein extraction; enzymatic extraction; antioxidant activity; ultrasound-assisted extraction

Bioactive compounds, such as proteins and polyphenols promoting the immune system, have huge importance for human nutrition. These kinds of compounds with beneficial effects on human health are known as "functional compounds" and they exhibit some nutritional properties such as reduction of oxidative stress, prevention of cancer, arteriosclerosis, ageing processes due to their antioxidant activity [1]. Among these compunds, proteins have also techno-functional properties such as solubility, foaming or emulsifying ability and they are used as emulsifying agents, texture modifiers and/or water/oil absorption enhancers by food industry [1–4].

On the other hand, as much as 75 % of the world's freshwater resources is necessary to produce traditional plant- and animal-based proteins [5]. Moreover, the COVID-19 pandemic has led to an increase in health awareness and demand for development of innovative bioactive compounds from various bioresources. Therefore, there is an urgent need to find alternative plant- and animal-based protein sources (e.g., microalgae, macroalgae, duckweed, insects or rapeseed) to traditional protein sources (e.g. soybean, chickpea, bean, lentil, pea, or lupin). Especially, to find cheap and sustainable protein resources and to valorize food processing by-products are the trending research topics in food science and technology [1, 6, 7].

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Macroalgae contain various bioactive components such as proteins, polyphenolic compounds and unsaturated fatty acids, offering the opportunity to obtain natural antioxidants from them. There has been important recent interest in the commercial utilization of macroalgae based on the contents of these valuable compounds with applications in the food industry. However, apart from direct use as food, the commercial exploitation of macroalgae has so far been limited to the production of agar and carrageenan, these compounds being used as thickening agents in various food products [8]. Moreover, there are commercial uses of algae as animal feed due to their high protein content [9].

Caulerpa prolifera, which is a macroalga of the family Caulerpaceae, is a great source for valuable compounds for biotechnological application. Several studies were published on extraction of proteins, phenolics and polysaccharides from it. For example, polysaccharides from *C. prolifera* demonstrated promising antioxidant, anti-proliferative and/or anticoagulant potential in the study of Costa et al. [10]. In the study of Caronni et al. [11], the simplest and least expensive extraction protocols among five conventional extraction methods were investigated for protein extraction from *C. prolifera*. Moreover, *Caulerpa* sp. was successfully incorporated as a functional ingredient into several foods at the laboratory scale in previous studies. For instance, in a study of Kumar et al. [12], *C. racemosa* was added to semi-sweet biscuits and its effect was investigated on nutritional, physical, antioxidative and sensorial characteristics of biscuits. According to Agusman and Wahyuni [13], *Caulerpa* sp. noodles could be a nutritional benefit for daily diets since they contain a certain amount of phenolic compounds.

New food technologies for the extraction of bioactive compounds such as proteins and polyphenols are emerging. Conventional extraction methods with low extraction efficiency, such as solid-liquid extraction, maceration or heat reflux, have many disadvantages, including extensive use of organic solvents with toxic effects, as well as high energy and time consumption. Moreover, conventional extraction may lead to reduction of bioactivity of functional components in foods due to their degradation during thermal treatment [4]. On the other hand, non-thermal novel extraction techniques provide a high extraction efficiency, use of low amounts of solvents, lesser time and energy [14]. Alternatively, membrane separation techniques like microfiltration, ultrafiltration or reverse osmosis are promising extraction methods [9]. In ultrasound-assisted extraction, a novel extraction method, acoustic cavitation has a destructive effect on cell wall of tissues and pours the matrix component into the solvent medium at shorter time and lower solvent consumption [14]. Similarly, enzyme-assisted extraction is an effective extraction method for bioactive compounds from natural sources due to enzymatic degradation of cell walls of tissues [15].

To the best of our knowledge, there has not yet been any study on the use of a carbohydrase enzyme and ultrasound waves as a pre-treatment to extract proteins from *C. prolifera* and on techno-functional properties of *C. prolifera* proteins. Moreover, to our knowledge, this is the first report investigating the industrial potential of *C. prolifera* proteins in terms of their techno-functionality. Therefore, the aims of this study were (i) to optimize the conditions of ultrasound-assisted enzymatic extraction of proteins from *C. prolifera* collected from Mediterranean coasts of Turkey by using response surface methodology (RSM) based on Box-Behnken design, (ii) to investigate the effect of extraction conditions on protein content (*PC*), total phenolics content (*TPC*) and antioxidant activity (*AOA*), (iii) to determine techno-functional properties of the proteins obtained under optimum extraction conditions and (iv) to reveal the potential of the protein extracts with techno-functional characteristics for industrial applications.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Hydrochloric acid, trichloroacetic acid (TCA), sodium hydroxide, gallic acid, potassium persulfate, bovine serum albumin, sodium carbonate, copper (II) chloride solution, neocuproine, ammonium acetate buffer, sodium citrate buffer, sodium acetate buffer, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, ethanol and hemicellulase enzyme (EC number: 232-799-9) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). All of the solvents and chemicals used were of analytical grade.

Collection of samples

Caulerpa prolifera was collected from the Mediterranean coast of Turkey (36°27'40.47"N, 30°32'38.18"E). The collected macroalgae samples were cleaned from their epiphytes and dried in a shaded area at 35 °C. The dried algae

were ground into powder particles using a laboratory type grinder Waring 8011 Eb Blender (Cole-Parmer, Vernon Hills, Illinois, USA) and sieved using a sieve. The powdered *C prolifera* (PCP) with < 500 μm particle diameter were packaged appropriately to avoid exposition to sunlight and oxygen, and stored at –20 °C for a maximum of 5 days.

Protein solubility determination

The protein solubility assay was performed according to the method of Morr et al. [16]. Briefly, approximately 0.75 g of PCP was mixed with 100 ml 0.1 mol·l^{-1} NaCl solution and this mixture was stirred to form a smooth paste. The pH of solution was adjusted to pH values ranged from 2.0 to 13.0 using 0.l mol·l^{-1} HCl or 0.1 mol·l^{-1} NaOH solution. The dispersion was stirred for 1 h under these conditions. Afterwards, the dispersion was centrifuged at 3 000 ×g and 4 °C for 15 min. Then, protein content (*PC*) of supernatant was determined using the method of Lowry et al. [17]. Bovine serum albumin was used as standard protein. The protein solubility (*PS*) of PCP was calculated using Eq. 1 and expressed in percent:

$$PS = \frac{c \times 50}{w \times PC} \times 100 \quad (1)$$

where c is concentration of supernatant (in milligrams per millilitre), w is weight of sample (in milligrams), *PC* is protein content, number 50 is dilution factor.

ζ-Potential

The ζ-potential of the samples was determined as a function of pH ranging from 2.0 to 13.0 using Zetasizer NanoZS90 (Malvern Instruments, Malvern, United Kingdom). Briefly, 1 g of PCP was mixed with distilled water and pH was adjusted to desired value using 0.1 mol·l^{-1} HCl or 0.1 mol·l^{-1} NaOH. The electrical charge (ζ-potential) was determined by measuring the direction and velocity with which the droplets moved in the applied electric field. The Smoluchowsky mathematical model was used by Maple software (Maplesoft, Duxford, United Kingdom) to convert the electrophoretic mobility measurements into ζ-potential values. All measurements were made from two freshly prepared samples and were carried out with three readings per sample.

Optimization of protein extraction

Extraction of proteins from PCP was carried out according to the method of Naseri et al. [18] with some modifications. Firstly, an aliquot of 1 g of PCP was combined with 30 ml citrate buffer (pH 4.5). Then, the suspensions were subjected to ultrasonic sonication through probe at 65% amplitude and 53 kHz frequence for 60 s by using an ultrasound homogenizer Sonopuls HD 2200 (Bandelin Electronic, Berlin, Germany). During sonication, the samples were cooled in an ice bath to avoid sample heating. After ultrasonic treatment, hemicellulase enzyme was added to the samples according to substrate/enzyme (S/E) ratios in the experimental design consisting of a total of 17 runs (samples; Tab. 1), placed in a shaking water bath NB-303 (N-Biotek, Gyeonggi-do, Korea) adjusted to the desired temperature, and allowed to react throughout certain extraction time determined by RSM (Tab. 1). After extraction, samples were kept in the water bath at 85 °C for 10 min for enzyme inactivation. Subsequently, to increase *PC*, pH value of the mixtures was adjusted to 10, which is one of the pH values causing high *PS*, and the mixtures were subjected to second extraction at 45 °C for 60 min in the shaking water bath. Then, the samples were centrifuged at 3 000 ×g and 4 °C for 30 min. The collected supernatants were stored at –20 °C in the dark until further analysis. All the following experimental results in this study were expressed by the dry weight (dw) of the sample.

Protein content

PC of the extracts was determined using the modified Lowry method (TCA-Lowry), which includes precipitation of proteins from the samples with TCA to remove potential interfering substances [19]. According to the method of Moein et al. [19], firstly, 1 ml of the protein extracts was added to 3 ml of 25% TCA solution and incubated in the shaking water bath at 4 °C for 30 min. Afterwards, the sample was centrifuged at 3 000 ×g for 20 min and the supernatant was withdrawn. The process was repeated sequentially by adding 10% TCA and 5% TCA solutions to the sediments. Finally, 2 ml of 0.1 mol·l^{-1} NaOH solution was added to the remaining precipitate and *PC* of the extracts was measured spectrophotometrically using an UV spectrophotometer Scilogex Sci-UV1000 (Scilogex, Rocky Hill, Connecticut, USA) according to the method of Lowry et al. [17]. Bovine serum albumin was used as the standard and *PC* was expressed as grams per kilogram sample dw.

Total phenolics content

TPC of the extracts was determined according to the Folin-Ciocalteu method [20]. Briefly, 200 μl of the extracts with 1.5 ml Folin-Ciocalteu reagent-water (1 : 10, v/v) and 1.2 ml of aqueous 7.5% Na_2CO_3 were mixed and allowed to stand

Tab. 1. Box-Behnken experimental design with natural and coded extraction conditions and experimentally obtained values of all investigated responses.

Run	Independent variables						Responses			
	Temperature [°C]		Time [min]		S/E		*PC* [g·kg^{-1}]	*TPC* [g·kg^{-1}]	AOA_{CUPRAC} [g·kg^{-1}]	AOA_{DPPH} [g·kg^{-1}]
1	1	40	0	90	–1	5	49.47	29.05	4.43	0.71
2	0	35	1	120	–1	5	59.42	19.91	3.76	0.44
3	0	35	0	90	0	10	17.59	13.24	2.21	0.59
4	0	35	–1	60	–1	5	48.54	28.32	4.31	0.71
5	0	35	1	120	1	15	20.60	14.05	3.04	0.70
6	–1	30	0	90	1	15	24.19	14.97	2.35	0.61
7	–1	30	0	90	–1	5	48.37	28.81	3.95	0.67
8	–1	30	1	120	0	10	30.34	18.39	2.90	0.73
9	1	40	–1	60	0	10	28.15	16.60	2.64	0.64
10	0	35	0	90	0	10	27.32	14.57	2.70	0.57
11	1	40	1	120	0	10	23.65	13.83	2.44	0.66
12	0	35	–1	60	1	15	16.49	8.25	1.39	0.69
13	–1	30	–1	60	0	10	32.53	16.37	2.60	0.82
14	0	35	0	90	0	10	26.57	14.03	2.33	0.70
15	1	40	0	90	1	15	26.04	14.79	2.83	0.72
16	0	35	0	90	0	10	24.90	12.18	2.20	0.75
17	0	35	0	90	0	10	24.73	13.42	2.43	0.67

S/E – substrate/enzyme ratio, *PC* – protein content expressed as grams of protein per kilogram of sample dry weight (dw), *TPC* – total phenolics content expressed as grams of gallic acid equivalents per kilogram of sample dw, AOA_{CUPRAC} – antioxidant activity determined by cupric reducing antioxidant capacity (CUPRAC) method and expressed as grams of Trolox equivalents (TE) per kilogram of sample dw, AOA_{DPPH} – antioxidant activity determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and expressed as grams of TE per kilogram of sample dw.

at room temperature (20–22 °C) in the dark for 90 min. The absorbance was read at 765 nm using the UV spectrophotometer Scilogex Sci-UV1000. *TPC* was calculated from a calibration curve using gallic acid as a standard. The results were expressed as grams of gallic acid equivalents (GAE) per kilogram of sample dw.

Antioxidant activity

CUPRAC method

The cupric reducing antioxidant capacity (CUPRAC) assay was carried out to determine antioxidant activity (AOA_{CUPRAC}) of the extracts as previously described [21]. In brief, 100 μl of the extracts was mixed with 1 ml each of 0.1 mmol·l^{-1} copper (II) chloride solution, 7.5 mmol·l^{-1} neocuproine solution, ammonium acetate buffer solution (pH 7.0) and distilled water. After 30 min of incubation at room temperature (20–22 °C), absorbance of the mixture was measured at 450 nm using the UV spectrophotometer Scilogex Sci-UV1000. Trolox was used as a standard and the results were expressed in grams of Trolox equivalents (TE) per kilogram of sample dw.

DPPH method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was carried out according to the method of Kumaran and Karunakaran [22]. Briefly, 100 μl of the extracts was mixed with 2 ml of 0.1 mmol·l^{-1} DPPH in methanol. The mixture was mixed by vortex and left to stand for 30 min in a dark place at room temperature (20–22 °C). Then, absorbance of the mixture was measured at 517 nm using the UV spectrophotometer Scilogex Sci-UV1000. The antioxidant activity (AOA_{DPPH}) were expressed in grams of TE per kilogram of sample dw.

Techno-functional properties

Water absorption capacity

Water absorption capacity (*WAC*) was determined according to the method of Kumar et al. [23]. Briefly, 0.1 g of the protein extract obtained under optimum extraction conditions was diluted with 10 ml distilled water and mixed for 30 s by a vortex mixer. The mixture was held at room temperature (20–22 °C) for 30 min and centrifuged at 3 000 ×*g* for 20 min. The supernatant was removed and the centrifuge tube containing sediment was

weighed. *WAC* was calculated using Eq. 2 and expressed in percent:

$$WAC = \frac{W_2 - W_1}{W_0} \times 100 \quad (2)$$

where W_0 is weight of protein extract, W_1 is weight of the tube plus protein extract and W_2 is weight of the tube plus the sediment (all expressed in grams).

Oil absorption capacity

Oil absorption capacity (*OAC*) was determined according to the method of Kumar et al. [23]. Briefly, 1.0 g of the protein extract was dispersed in 5 ml of sunflower oil and centrifuged at 3 000 ×*g* for 20 min. The supernatant was discharged and the tubes were weighed. *OAC* was calculated using Eq. 3 and expressed in percent:

$$OAC = \frac{O_2 - O_1}{O_0} \times 100 \quad (3)$$

where O_0 is weight of the sample, O_1 is weight of the tube plus protein extract and O_2 is weight of the tube plus the sediment (all expressed in grams).

Emulsifying activity and emulsifying stability

Emulsifying activity (*EA*) and emulsifying stability (*ES*) were determined using the methods of Kumar et al. [23]. Briefly, 0.1 g of the sample was dissolved in 10 ml of distilled water and homogenized for 2 min at room temperature (20–22 °C) using a hand-held homogenizer MT-30K MIULAB (Hangzhou Miu Instruments, Zhejiang, China). After the homogenization, 10 ml of sunflower oil was added to the mixture and homogenized again under the same conditions. Then, the mixture was centrifuged at 3 000 ×*g* for 5 min. The height of the emulsion layer was recorded and *EA* of the samples was calculated using Eq. 4 and expressed in percent:

$$EA = \frac{HE}{HC} \times 100 \quad (4)$$

where *HE* is height of emulsified layer and *HC* is height of contents of the tube.

In the *ES* assay, the samples were heated at 80 °C for 30 min, then centrifuged at 1200 ×*g* for 5 min. *ES* of the samples was calculated using Eq. 5 and expressed in percent:

$$ES = \frac{HR}{HO} \times 100 \quad (5)$$

where *HR* is height of remaining emulsified layer, *HO* is height of original emulsified layer.

Foaming capacity and foaming stability

Foaming capacity (*FC*) and foaming stability (*FS*) of the extracts were determined according to the methods of Kumar et al. [23]. Briefly, 0.02 mg of the protein extract was added to 20 ml of distilled water and whipped for 2 min using the hand held homogenizer.

FC was calculated using Eq. 6 and expressed in percent.

$$FC = \frac{VW_0 - VW_1}{VW_0} \times 100 \quad (6)$$

where VW_0 is volume before whipping and VW_1 is volume after whipping (all expressed in millilitres).

After the homogenization, the mixture was kept at room temperature (20–22 °C) for 30 min. *FS* was calculated using Eq. 7 and expressed in percent.

$$FS = \frac{VW_2 - VW_0}{VW_0} \times 100 \quad (7)$$

where VW_2 is volume after standing and VW_0 is volume before whipping (all expressed in millilitres).

Experimental design statistical analysis

RSM was used for optimization of three extraction parameters on three levels. A Box-Behnken design with 5 central points was employed. In this study, independent variables (extraction temperatures of 30 °C, 35 °C and 40 °C, extraction times of 60 min, 90 min and 120 min and S/E ratios of 5, 10 and 15; Tab. 2) were used in the experimental design. Extraction parameters were normalized as coded variables. Variables were coded according to Eq. 8:

$$X = \frac{x_i - x_0}{\Delta x} \quad (8)$$

where x_i is corresponding actual value, x_0 is actual value in the centre of the domain and Δx is increment of x_i corresponding to a variation of one unit in *X*.

The response functions (*Y*) were *PC*, *TPC* and *AOA*. The response variables were fitted to

Tab. 2. Experimental and coded levels of independent variables for Box-Behnken Design.

Independent variables	Coded levels		
	–1	0	1
	Natural levels		
Temperature [°C]	30	35	40
Time [min]	60	90	120
Substrate/enzyme ratio	5	10	15

a second-order polynomial model to obtain the regression coefficients (β). The generalized second-order polynomial model used in the response surface analysis was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1 + \beta_{22} X_2 + \\ + \beta_{33} X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \quad (9)$$

where β_0 is constant term, β_i are linear effects, β_{ii} are quadratic effects, β_{ij} are interaction effects and ε is random error term that represents the variability of the response.

To evaluate model adequacy, regression coefficients and statistical significance, analysis of variance (ANOVA) was used. To visualize the relationships between the responses and the independent variables, surface response and contour plots of the fitted polynomial regression equations, as well as optimal conditions for the targeted responses were generated using the trial version of Design Expert 7.1 software (Stat-Ease, Minneapolis, Minnesota, USA). The results were statistically tested at a significance level of $p = 0.05$. The adequacy of the model was determined using model analysis, coefficient of determination (R^2) and lack-of-fit testing. A mathematical model was established to describe the influence of a single process parameter and/or the interaction of multiple parameters on each investigated response.

RESULTS AND DISCUSSION

Model fitting

PC, *TPC* and *AOA* were determined as functions of linear, quadratic and interaction terms of the independent variables including extraction temperature, extraction time and S/E using Box-Behnken design. Results of analysis of variance and R^2 for each dependent variable are presented in Tab. 3. R^2 values were 0.92, 0.99, 0.95 and 0.50 for *PC*, *TPC*, AOA_{CUPRAC} and AOA_{DPPH}, re-

Tab. 3. Analysis of variance of the fitted second-order polynomial model.

PC

Source		Sum of squares	*DF*	Mean square	*F*-value	*p*-value
Model		2291.25	9	254.58	8.45	0.0051 *
Linear	β_1	8.24	1	8.24	0.27	0.6171
	β_2	8.61	1	8.61	0.29	0.6096
	β_3	1754.82	1	1754.82	58.23	0.0001 *
Quadratic	β_{11}	28.47	1	28.47	0.94	0.3634
	β_{22}	14.31	1	14.31	0.47	0.5129
	β_{33}	437.42	1	437.42	14.51	0.0066 *
Interaction	β_{12}	1.34	1	1.34	0.05	0.8388
	β_{13}	0.14	1	0.14	0.00	0.9471
	β_{23}	11.49	1	11.49	0.38	0.5565
Residual		210.95	7	30.14	–	–
Lack of fit		151.11	3	50.37	3.37	0.1358
Pure error		59.85	4	14.96	–	–
Corrected total sum of squares		2502.20	16	–	–	–

$R^2 = 0.9158$, $CV = 17.6$ %

TPC

Source		Sum of squares	*DF*	Mean square	*F*-value	*p*-value
Model		586.03	9	65.11	68.58	< 0.0001 *
Linear	β_1	2.28	1	2.28	2.40	0.1650
	β_2	1.40	1	1.40	1.48	0.2638
	β_3	364.81	1	364.81	384.23	< 0.0001 *
Quadratic	β_{11}	52.82	1	52.82	55.63	0.0001 *
	β_{22}	2.25	1	2.25	2.37	0.1678
	β_{33}	100.04	1	100.04	105.36	< 0.0001 *
Interaction	β_{12}	5.74	1	5.74	6.04	0.0436 *
	β_{13}	0.05	1	0.05	0.05	0.8308
	β_{23}	50.51	1	50.51	53.20	0.0002 *
Residual		6.65	7	0.95	–	–
Lack-of-fit		3.42	3	1.14	1.41	0.3628
Pure error		3.23	4	0.81	–	–
Corrected total sum of squares		592.68	16	–	–	–

$R^2 = 0.9888$, $CV = 5.7$ %

Tab. 3. *continued*

***AOA*$_{CUPRAC}$**

Source		Sum of squares	*DF*	Mean square	*F*-value	*p*-value
Model		10.12	9	1.12	14.18	0.0010 *
Linear	β_1	0.04	1	0.04	0.45	0.5221
	β_2	0.18	1	0.18	2.27	0.1760
	β_3	5.85	1	5.85	73.84	< 0.0001 *
Quadratic	β_{11}	0.30	1	0.30	3.84	0.0908
	β_{22}	0.01	1	0.01	0.00	0.9776
	β_{33}	2.36	1	2.36	29.74	0.0010 *
Interaction	β_{12}	0.06	1	0.06	0.81	0.3979
	β_{13}	0.00	1	0.00	0.00	0.9995
	β_{23}	1.21	1	1.21	15.25	0.0059 *
Residual		0.55	7	0.08	–	–
Lack of fit		0.38	3	0.13	3.01	0.1572
Pure error		0.17	4	0.04	–	–
Corrected total sum of squares		10.67	16			

$R^2 = 0.9480$, $CV = 9.9$ %

***AOA*$_{DPPH}$**

Source		Sum of squares	*DF*	Mean square	*F*-value	*p*-value
Model		0.06	9	0.01	0.78	0.6424
Linear	β_1	0.00	1	0.00	0.11	0.7445
	β_2	0.01	1	0.01	1.62	0.2433
	β_3	0.01	1	0.00	0.58	0.4700
Quadratic	β_{11}	0.00	1	0.00	1.20	0.3090
	β_{22}	0.00	1	0.00	0.07	0.7951
	β_{33}	0.00	1	0.01	0.47	0.5141
Interaction	β_{12}	0.00	1	0.00	0.41	0.5408
	β_{13}	0.00	1	0.00	0.16	0.7045
	β_{23}	0.02	1	0.02	2.47	0.1604
Residual		0.06	7	0.01	–	–
Lack-of-fit		0.03	3	0.01	1.98	0.2588
Pure error		0.02	4	0.01	–	–
Corrected total sum of squares		0.11	16			

$R^2 = 0.5015$, $CV = 13.3$ %

PC – protein content expressed as grams of protein per kilogram of sample dry weight (dw), *TPC* – total phenolics content expressed as grams of gallic acid equivalents per kilogram of sample dw, *AOA*$_{CUPRAC}$ – antioxidant activity determined by cupric reducing antioxidant capacity (CUPRAC) method and expressed as grams of Trolox equivalents (TE) per kilogram of sample dw, *AOA*$_{DPPH}$ – antioxidant activity determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and expressed as grams of TE per kilogram of sample dw, DF – degrees of freedom, R^2 – coefficient of determination, CV – coefficient of variance.
* – significant at $p \leq 0.05$.

Tab. 4. Response surface models for process conditions of protein extraction from *Caulerpa prolifera*.

Response	Quadratic polynomial model
Y_1	$Y_1 = 222.97750 - 7.21220X_1 - 0.086883X_2 - 10.36520X_3 - (3.850000 \times 10^{-3})X_1X_2 + (7.50000 \times 10^{-3})X_1X_3 - - 0.022283X_2X_3 + 0.10401X_1^2 + (2.05028 \times 10^{-3})X_2^2 + 0.40781X_3^2$
Y_2	$Y_2 = 213.11362 - 9.26213X_1 + 0.17478X_2 - 7.23085X_3 - (7.98533 \times 10^{-3})X_1X_2 + (4.32199 \times 10^{-3})X_1X_3 + + 0.023690X_2X_3 + 0.14167X_1^2 - (8.11889 \times 10^{-4})X_2^2 + 0.19497X_3^2$
Y_3	$Y_3 = 20.00798 - 0.66357X_1 - (2.88632 \times 10^{-3})X_2 - 1.09964X_3 - (8.44894 \times 10^{-4})X_1X_2 + (3.60011 \times 10^{-6})X_1X_3 + + (3.66538 \times 10^{-3})X_2X_3 + 0.010757X_1^2 + (4.43331 \times 10^{-6})X_2^2 + 0.029928X_3^2$
Y_4	$Y_4 = 4.38244 - 0.15941X_1 - X_2 - 0.037866X_3 + (1.90555 \times 10^{-4})X_1X_2 + (7.02866 \times 10^{-4})X_1X_3 + + (4.65453 \times 10^{-4})X_2X_3 + (1.90149 \times 10^{-3})X_1^2 + (1.29900 \times 10^{-5})X_2^2 - (1.19112 \times 10^{-3})X_3^2$

Y_1 – protein content, Y_2 – total phenolics content, Y_3 – antioxidant activity (determined by CUPRAC method), Y_4 – antioxidant activity (determined by DPPH method), X_1 – extraction temperature, X_2 – extraction time, X_3 – substrate/enzyme ratio.

spectively (Tab. 3). The variation coefficient (*CV*) of the model can be low as an indication of good reproducibility of the investigated systems. However, unlike *PC* (*CV* = 17.6 %) and AOA_{DPPH} (*CV* = 13.3 %), *TPC* (*CV* = 5.7 %) and AOA_{CUPRAC} (*CV* = 9.9 %) showed low variation in their mean values. The lack-of-fit was not significant for *PC*, *TPC*, AOA_{CUPRAC} and AOA_{DPPH} ($p > 0.05$). These results demonstrated that the model for *PC* ($p = 0.0051$), *TPC* ($p < 0.0001$) and AOA_{CUPRAC} ($p = 0.0010$) can be used to optimize the extraction parameters for *C. prolifera* proteins. Data on quadratic models for all responses are shown in Tab. 4.

Protein solubility and ζ-potential

Fig. 1 shows the changes in *PS* and ζ-potential of PCP at various pH ranging from 2.0 to 12.0 with 1.0 interval. ζ-Potential of PCP was negative at all pH values, being the closest to zero at pH 2.0 (–14.03 ± 0.02 mV). The cell wall of macroalgae is a double-layered structure composed of lipids and proteins. Proteins make up a small part of the structure while phospholipids make up the majority. Phosphate groups on the outside of phospholipids can become negatively charged in seawater. Therefore, the negative charges can be much more numerous, affect the surface charge and the negative charges of polysaccharides in the extracts cause negative ζ-potential value [24].

PS of PCP was the lowest at pH 3.0 (1.3 ± 0.1 %), whereas it showed the highest solubility at pH 12.0 (3.5 ± 0.1 %). According to Ursu et al. [25], proteins exhibit high solubility under alkaline pH values because of their net electrical charges. There is no study about protein solubility and ζ-potential of *C. prolifera* in the literature. In algae similar to *C. prolifera*, the highest value for *PS* of *Chlorella vulgaris* and *Phaeodactylum tricornutum* was reached at pH 12.0 [25].

Protein content of the extracts

As shown in Tab. 1, the highest *PC* was obtained as 59.42 g·kg^{-1} under the applied extraction conditions (extraction temperature of 35 °C, extraction time of 120 min and S/E of 5). The linear effect ($p = 0.0001$) and quadratic effect ($p = 0.0066$) of S/E were significant on *PC* of *C. prolifera* protein extracts (CPPE). These results are consistent with the observation of Harnedy and Fitzgerald [15] who reported that the utilization of polysaccharidase to break down cell wall increased the extraction of proteins from macroalgae. Algal cell wall was destroyed by the used enzyme, thus more protein was released into the solvent medium. Similarly, Vásquez et al. [26] investigated the effects of enzymatic and non-enzymatic methods on protein extraction from brown macroalgae *Macrocystis pyrifera* and red macroalgae *Chondracanthus chamissoi*. They found that the disruption of the cellulase-sensitive carbohydrate matrix increased *PC* of the extract [26]. Similarly, Joubert and Fleurence [27] investigated that the effect of xylanase and cellulase enzymes and enzyme concentration on *PC* of *Palmaria palmata*. They reported that *PC* increased as the amount of enzyme increased [27].

Total phenolics content of the extracts

TPC of CPPE varied from 8.25 g·kg^{-1} to 29.05 g·kg^{-1} (expressed as GAE) under the extraction conditions given in Tab. 1. Similarly, *TPC* of *Caulerpa racemosa* observed in the ethyl acetate extract was 17.88 ± 0.78 g·kg^{-1} [28]. However, the highest content of polyphenol extracts obtained from *Caulerpa lentillifera* using ethanol solvent technique was determined as 73.00 ± 2.08 g·kg^{-1} in the study conducted by Wichachucherd et al. –29]. Moreover, Caronni et al. [11] determined *TPC* of 9.29 g·kg^{-1} (expressed as phloroglucinol equivalent), fort protein extracts from *C. prolifera*. On the other hand, *TPC* of the ethanolic extract of *C. lentillifera* was 1.30 g·kg^{-1} (expressed as GAE) [30]. In food incorporation studies on *Caulerpa* sp., for instance, with an increase in the content of *C. racemosa* in biscuits, *TPC* and antioxidant activity increased [12].

As shown in Tab. 3, linear effect of S/E ($p < 0.0001$) and quadratic effect of extraction temperature ($p < 0.0001$) and S/E ($p = 0.0001$) were significant on *TPC* of the extracts. When the algal cell wall is destroyed, internal components, such as proteins, are liberated, which tend to bind to polyphenols, resulting in aggregation and pre-

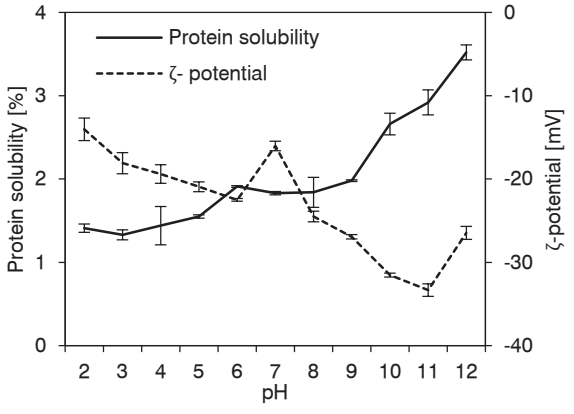

Fig. 1. Protein solubility and ζ-potential of *C. prolifera* macroalgae at various pH.

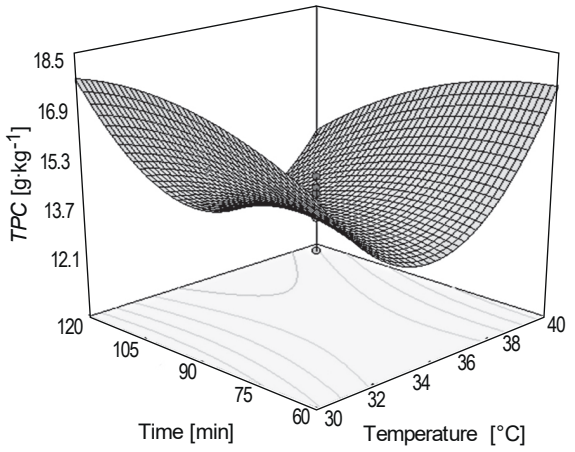

Fig. 2. 3D contour plot response surface for the effect of cross-interaction between extraction time and extraction temperature on total phenolic content.

TPC – total phenolics content expressed as grams of gallic acid equivalents per kilogram of sample dry weight.

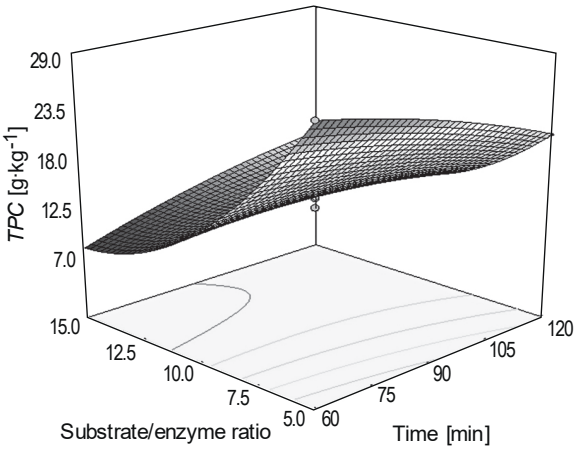

Fig. 3. 3D contour plot response surface for the effect of cross-interaction between extraction time and substrate/enzyme ratio on total phenolic content.

TPC – total phenolics content expressed as grams of gallic acid equivalents per kilogram of sample dry weight.

cipitation. Potentially, polyphenols may interact with proteins via hydrogen bonding, p-bonding, hydrophobic interactions, ionic and covalent linkage [31]. Similar to *PC*, *TPC* of the extracts increased with an increase in the enzyme amount, because significant quantities of proteins are found bound to other non-protein components like polyphenols within algal cells [15].

According to the RSM analysis, the interaction effects between extraction temperature and time ($p = 0.0436$), as well as between extraction time and S/E ($p = 0.0002$), were significant on *TPC* of the extracts (Tab. 3). The graph plot revealed that maximum *TPC* in the extracts was obtained under extraction time ranging from 60 min to 120 min and extraction temperature of approximately 30 °C, as well as under extraction time ranging from 60 min to 105 min and extraction temperature of approximately 38–40 °C (Fig. 2). Additionally, an increase in *TPC* of the extracts was recorded at S/E of approximately 5 and the extraction time of 60–90 min (Fig. 3).

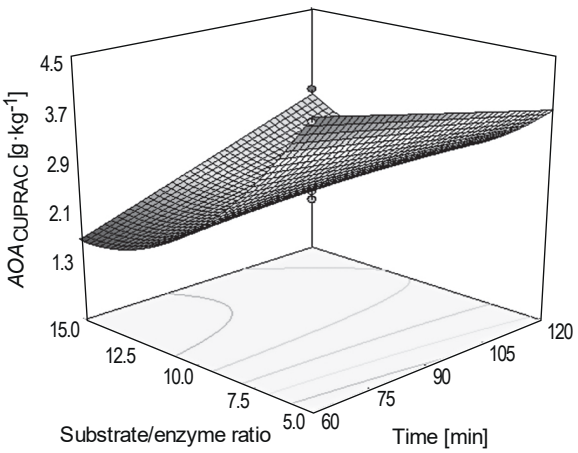

Fig. 4. 3D contour plot response surface for the effect of cross-interaction between extraction time and substrate/enzyme ratio on antioxidant activity.

*AOA*CUPRAC – antioxidant activity determined by cupric reducing antioxidant capacity assay.

Antioxidant activity of the extracts

*AOA*CUPRAC and *AOA*DPPH of CPPE (expressed as TE) ranged from 1.39 g·kg^{-1} to 4.43 g·kg^{-1} and from 0.44 g·kg^{-1} to 0.82 g·kg^{-1}, respectively (Tab. 1). Similarly, *AOA* values of *Sargassum wightii*, *Ulva rigida* and *Gracilaria edulis* were previously determined as 8.21 g·kg^{-1}, 6.90 g·kg^{-1} and 1.06 g·kg^{-1}, respectively [32]. On the other hand, in the study of Wichachucherd et al. [29], the highest *AOA* for *C. lentillifera* was determined as 29.5 ± 0.8 %. These variations in *AOA* could be attributed to harvest season, harvest time, geographical location and algal species [29].

The linear effect of S/E ($p < 0.0001$), quadratic effect of S/E ($p = 0.0010$) and interaction effect between extraction time and S/E ($p = 0.0059$) were significant on *AOA*CUPRAC. Similarly, in the study of Wang et al. [33], all commercial enzymes used were effective on extraction of antioxidant ingredients from red algae *Palmaria palmata*. Fig. 4 shows that *AOA* of the extracts was the highest when the extraction time was 60–90 min and S/E was approximately 5. Similar to *TPC*, *AOA* of the

extracts increased with an increase in the enzyme amount used for extraction.

Optimization and verification

Optimization procedures were performed to predict the optimum level of independent variables. The optimum extraction conditions were as follows: extraction temperature of 30 °C, extraction time of 60.03 min and S/E of 5. Predicted and mean experimental values for *TPC* (31.12 g·kg^{-1} dw and 27.05 ± 1.52 g·kg^{-1} dw), *AOA*$_{\text{CUPRAC}}$ (4.45 g·kg^{-1} and 4.19 ± 0.13 g·kg^{-1}), *AOA*$_{\text{DPPH}}$ (0.83 g·kg^{-1} and 0.80 ± 0.01 g·kg^{-1} dw) and *PC* (51.57 g·kg^{-1} dw and 50.66 ± 4.02 g·kg^{-1} dw) indicated that the differences between the predicted and mean experimental values were not significant ($p \geq 0.05$).

Techno-functional properties

In addition to the bioactive properties of proteins, their techno-functional properties such as *WAC*, *OAC*, *FC, FS*, *EA* or *ES*, should also be investigated since proteins as food additives have important effects on physico-chemical properties of food. In the present study, *WAC*, *OAC*, *FC, FS*, *EA,* and *ES* values of CPPEs obtained under optimum extraction conditions were determined.

Water and oil absorption capacities

WAC of proteins is one of the most important characteristics for various foods such as soups or dough [34]. Proteins can be added to foods to improve mouthfeel, thickness and viscosity of foods. In the present study, *WAC* of CPPE obtained under optimum extraction conditions was 54.0 ± 5.3 %. Unlike the present work, *WAC* values were previously determined as 153.0 ± 7.0 % for *Enteromorpha compressa*, 132.0 ± 11.0 % for *E. tubulosa*, 122.0 ± 6.0 % for *E. linza* [34] and 222.0 ± 4.0 % for *Kappaphycus alvarezii* [23]. The *WAC* values of algal proteins determined by previous studies were higher than those obtained in the present study. This may be due to the fact that proteins from *C. prolifera* were extracted at an alkaline pH value and its *PS* was high under alkaline conditions according to the results of *PS* assay (Fig. 1). Therefore, the proteins dissolved easily and soluble proteins were discarded with supernatant during *WAC* assay. Similarly, according to Chen et al. [35], *WAC* value increased with a decrease in *PS* due to more phosphate groups and/or lower α-helix structures in proteins, which improve hydration of proteins. Moreover, the protein structure as well as hydrophobic and hydrophilic balance of amino acids affect the water-binding property of proteins [35]. According to the results of *WAC* analysis, CPPE may not be a suitable candidate to improve *WAC* of foods, because *WAC* values must be between 149.0 % and 472.0 % to improve foods in terms of water-holding property [35].

On the other hand, *OAC* is the ability of proteins to physically hold oil and indirectly affect flavour and texture of foods such as meat, sausages or mayonnaise [23]. In this work, *OAC* of CPPE was 210.6 ± 15.0 %. Previously, *OAC* of *K. alvarezii* was found to be 129.0 ± 20.0 % by Kumar et al. [23]. Also, Kandasamy et al. [34] determined *OAC* values of *E. compressa*, *E. tubulosa* and *E. linza* as 134.0 ± 10.0 %, 108.0 ± 4.0 % and 105.0 ± 7.0 %, respectively. The differences in values of *OAC* probably resulted from different amino acid composition and protein conformation of proteins, as well as extraction methods or their parameters.

OAC of *C. prolifera* proteins was higher than those of some macroalgae. Accordingly, CPPE with a notable *OAC*, as an oil holding agent, can be added to foods as and oil-holding substance or texture enhancer. For example, in the study of Kumar et al. [12], *C. racemosa* was used in biscuits as a substitute for refined flour at levels of 1.0 %, 5.0 %, and 10 %. With the increase in the content of *C. racemosa* in biscuits, water and oil absorption capacity of the flour mixture increased.

Emulsification and foaming properties

Other important factors determining the commercial value of food proteins are their emulsification and foaming properties.

The *EA* and *ES* terms are used to explain the emulsification properties of food proteins. *EA* is the capacity of proteins to assist in the formation and stabilization of the emulsion. On the other hand, *ES* is defined as a proteins' ability to stabilize an emulsion without affecting its structure over a period of time, while *EA* and *ES* of proteins depend on their hydrophobicity, net surface charge and solubility [2]. In the present work, *EA* and *ES* of CPPE were 41.4 ± 2. % and 41.7 ± 0.0 %, respectively. Likewise, EA and ES of *Spirulina platensis* proteins were found to be 40.0–46.0 % and 83.3–100.0 % by Yücetepe et al. [36]. The *EA* and *ES* values of CPPE were lower than those of commercial emulsifier agents. Emulsion capacities of lupin seed and soya bean, whose extracts are used as emulsifiers in food industry, were 60 % and 70 %, respectively [37].

Foaming properties are explained by *FC* and *FS*. *FC* is defined as the potential of a protein to form foam when gas is introduced into the protein solution, whereas *FS* is determined by measuring a decrease in foam volume over a measured

period of time [38]. In the present study, *FC* and *FS* of CPPE were determined as 26.5 ± 1.5 % and 26.7 ± 2.4 %, respectively. Compared to previous studies on various proteins, *FC* of CPPE was lower than that of protein concentrate from brown macroalgae *Himanthalia elongata* protein (71.5 ± 4.8 %) and *Kappaphycus alvarezii* protein (53.3 ± 2.3 %) [23, 39]. On the other hand, *FS* values of *E. tubulosa*, *E. compressa*, *E. linza* and *K. alvarezii* were found as 16.7 ± 1.5 %, 37.5 ± 2.0 %, 4.4 ± 2.0 %, and 45.3 ± 1.2 %, respectively [23]. A variety of factors, such as concentration, pH and temperature, as well as methods for foam production may cause the differences between foaming properties of various proteins [38].

CONCLUSION

The extraction conditions to obtain proteins from *C. prolifera* were successfully optimized by RSM. The optimum conditions were as follows: extraction temperature of 30 °C, extraction time of 60.03 min and substrate/enzyme ratio of 5. According to the result of the study, the carbohydrase enzyme increased *PC*, *TPC* and *AOA* of the extracts. Additionally, *C. prolifera* proteins may has a potential to be applied to oil-based food formulations due to their good level of *OAC*. However, more studies are needed on the sensory attributes of the final food products after incorporating the novel protein source.

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