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ORIGINAL ARTICLE

Ultrasound-assisted enzymatic extraction of proteins from *Gracilaria dura*: Investigation of antioxidant activity and techno-functional properties

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Abstract

In this study, ultrasound-assisted enzymatic extraction was performed to extract proteins from *Gracilaria dura* for the first time. The ultrasonic applying time (30–300 s), enzyme/substrate (E/S, 0.5–2.5), and extraction time (20–28 h) on protein content (PC), total phenolic content (TPC), and antioxidant activity (AOA) was optimized using response surface methodology. Techno-functional properties of protein extracts obtained under optimum conditions were determined. At optimum conditions (ultrasonic applying time: 257.57 s, E/S: 2.5, extraction time: 22.61 h), PC, TPC, AOA_{CUPRAC} and AOA_{ABTS} were found as 189.59 mg/g, 60.52 mg GAE/g and 55.66 mg TE/g and 478.50 mg TE/g dw, respectively. The water/oil absorption capacity, foaming capacity/stability, emulsifying activity/stability of the protein extracts were 195 ± 0.08%, 568 ± 0.10%, 12.5 ± 0.00%, 0%, 44 ± 0.00%, and 75 ± 2.50%, respectively. In conclusion, *G. dura* protein extracts may have an important potential to improve antioxidant activity and functional properties of various food products due to high antioxidant activity and a good level of water/oil absorption capacity.

Novelty impact statement: Macroalgae *Gracilaria dura* proteins were successfully extracted with high extraction efficiency by extraction process performed with ultrasonication application and enzyme usage. The protein extracts from *G. dura* can be used to improve the techno-functional and antioxidative properties of various food products due to their acceptable water/oil absorption capacity and emulsifying properties and high antioxidant activity.

1 | INTRODUCTION

There is a preference for natural food products and functional food ingredients, which are believed to benefit human health and exist inherently in foods, instead of receiving compounds chemically synthesized. Moreover, to strong immunity system is among people's highest priority in a post-pandemic era as the Covid-19 pandemic caused the catastrophic effects. In particular, bioactive extracts or single functional food components that can prevent nutrition-related diseases have huge importance for human nutrition and promote the human immune system (Galanakis, 2021). In this context, finding alternative, cheap, natural, and sustainable food resources such as macroalgae, duckweed, and insects for bioactive compounds is one of the trend research topics.

Macroalgae are renewable sources that live in the marine ecosystem and are excellent for human nutrition, also have an important commercial potential as a functional ingredient in a variety of foods (Cofrades et al., 2017; García-Vaquero et al., 2017). In particular, proteins as one of the essential nutrients isolated from macroalgae have various bioactive properties such as antioxidant, anticancer,

antiviral, and antibacterial activities. Macroalgae-derived proteins have also techno-functional properties such as foaming or emulsifying ability and they can be used as emulsifying agents, texture modifiers, and/or water/oil absorption enhancers by the food industry (Galanakis et al., 2021; Michalak & Chojnacka, 2015).

Although macroalgae include a variety of high-value compounds, the extraction of macroalgae-derived proteins is complicated due to several variables. The most important of these is the presence of complex cell wall polysaccharides (e.g., alginate, carrageenan, cellulose, hemicellulose) which can reduce the extraction effect (Bleakley & Hayes, 2017; Harnedy & FitzGerald, 2011; Kadam et al., 2013). In this context, in conventional extraction methods with low extraction yields, organic solvents that cause negative effects on the environment are used (Bleakley & Hayes, 2017). Therefore, there is a strong interest in the development of environmentally friendly novel extraction techniques (e.g., ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, etc.) to extract bioactive compounds with higher extraction yield and lower extraction time and costs (Cravotto & Binello, 2016; Kadam et al., 2013; Ochoa-Rivas et al., 2017; Tiwari, 2015). Additionally, membrane separation techniques such as microfiltration, ultrafiltration, and reverse osmosis are one of the methods which show great promise for isolating compounds (Bleakley & Hayes, 2017). Among these, ultrasound-assisted extraction leads to acoustic cavitation to break down cell wall polymers (Bleakley & Hayes, 2017). On the other part, in enzyme-assisted extraction, to dissolve polysaccharide cell walls, food-grade enzymes such as carbohydrase and protease enzymes are commonly utilized, which require little or no chemical solvents and also exhibit excellent catalytic efficiency, improve overall protein extraction yields. For instance, Naseri et al. (2020) used several enzymes such as Celluclast®, Shearzyme®, Alcalase®, and Viscozyme® to extract protein from *Palmaria palmata* and reported that protein yield ranged from 35.5% to 41.6%. Similarly, Vásquez et al. (2019) investigated the effects of enzymatic and non-enzymatic methods on protein extraction from macroalgae *Macrocystis pyrifera* and *Chondracanthus chamissoi*. They found that the disruption of the cellulase-sensitive carbohydrate matrix increased the protein content of the extract (Vásquez et al., 2019).

Macroalgae *Gracilaria* sp. has high protein content which varies between 5.6% and 24.0% (Francavilla et al., 2013; Friedlander, 2008; Gressler et al., 2010; Wen et al., 2006). In the study of Sambhwani et al. (2020), the highest protein content of *Gracilaria dura* was determined as 23.0% depending on seasonal conditions. To the best of our knowledge, there is no research on the combined effect of extraction time, enzyme/substrate ratio (E/S) and ultrasonic applying time on protein content (PC), total phenolic content (TPC), and antioxidant activity (AOA) of protein extracts from *G. dura*. Therefore, the aims of this study were to (i) determine the fatty acid, mineral, and carbohydrate profile of *G. dura* (ii), optimize the conditions of ultrasound-assisted enzymatic extraction of proteins from *G. dura* by using response surface methodology (RSM) (iii), investigate the effect of extraction conditions on PC, TPC, and AOA (iv), determine techno-functional properties and organic group with Fourier transform infrared (FT-IR) spectroscopy method of the proteins extracts obtained under optimum extraction conditions.

2 | MATERIAL AND METHOD

2.1 | Material

Gracilaria dura was collected from the Aegean coast of Turkey (coordinates: 40°1′35.90″ N and 26°19′49.49″ E). The collected macroalgae samples were washed with tap water and dried in a shaded area at ~30°C. The dried macroalgae were ground into powder particles using a laboratory-type grinder (Waring 8011 Eb Blender, Cole-Parmer Instrument Company) and sieved using a sieve with a mesh size of 500 μm. The powdered *G. dura* (PG) with <500 μm particle diameter were packaged appropriately to avoid sunlight and oxygen, and stored at −20°C until further analysis.

2.2 | Chemicals

Folin–Ciocalteu's phenol reagent was purchased from Merck (Merck). 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-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, methanol, and ethanol were procured from Sigma–Aldrich (Sigma–Aldrich Chemie). Hemicellulase enzyme (HSP 50000) was purchased from Bakezyme. All of the solvents and chemicals used were of the analytical grade.

2.3 | Proximate composition and fatty acid, carbohydrates, and mineral profile analysis

The ash (923.03), lipid (920.85), crude protein (N × 6.25) (920.87), crude fiber (978.10), and moisture content (950.46) of PG were determined according to the Association of Official Analytical Chemists methods (AOAC, 2003). The phenol-sulfuric acid assay was used to determine soluble carbohydrate content (Dubois et al., 1956).

2.3.1 | Fatty acid profile

Fatty acid methyl esters (FAMEs) compositions of PG were determined according to the method of Uluata et al. (2021). The FAMEs were analyzed using an Agilent 7820A (Agilent Technologies Inc.) gas chromatography equipped with a capillary column (30 m × 0.25 mm i.d., 0.25 μm; Agilent 112-8837), a flame ionization detector (FID), and a 16-sample automatic liquid sampler. The injector and detector temperatures were maintained at 250°C and

280°C, respectively. The injection volume was 1 μl. The carrier gas hydrogen flow rate was 40 ml/min, and the split ratio was 1/50. The retention time of each FAME was compared against the standard mixture of FAMEs to identify the FAME's composition of the sample. Fatty acid composition results were expressed as a weight percentage.

2.3.2 | Carbohydrates profile

Carbohydrate profile analysis of PG was carried out according to the method of Pfetzing et al. (2000) with slight modifications. Mannitol, myo-inositol, and simple carbohydrates were quantified in PG using high-performance anion-exchange chromatography (HPAEC). Briefly, 10 mg of PG was mixed with 3-ml ultra-pure water (Millipore Milli-Q®) to extract sugars from *G. dura*. After shaking the suspension for 4 h at 80°C, aliquots of the supernatant were taken and kept at −18°C for subsequent analysis. A Dionex ICS-5000 chromatography system with a PA-200 column and a guard column was used to perform HPAEC (Dionex Corporation). A gradient of Solution A (600 mM NaOH) and Solution B (100 mM NaAc in 600 mM NaOH solution) was used as mobile phases. The simple sugars of PG were separated using a binary gradient method: 0% B for 3 min, 12% B for 15 min, 0% B again in 12 min, and so on for a total of 30 min, with a flow rate of 0.40 ml/min and the temperature of 25°C. For detection, an electrochemical ED40 detector in integrated amperometric mode was used.

2.3.3 | Mineral profile

The PG, which was subjected to microwave digestion and filtered, was taken to the stage of determination of heavy metal contents in the inductively coupled plasma mass spectrometry (ICP-MS). For this purpose, first, blank and standard solutions were prepared in order to draw a calibration curve. Then, 2% HNO_3 solution, prepared from 65% HNO_3 (Suprapure) with ultrapure water, was used as blank. Afterward, standard concentrations ranged from 1 ppb to 200 ppb were prepared from 10 ppm pure standard that has equal concentrations of heavy metals, and the calibration curve was drawn by analyzing these standards (Ahamad et al., 2017; Pilarczyk et al., 2013).

2.4 | Protein solubility

The protein solubility assay of PG was carried out according to the method of Morr et al. (1985). Briefly, 1 g of PG was mixed with 50 ml of 0.1 N NaCl solution and the pH of the mixture was adjusted to the desired value ranging from 2 to 13 (with a 1.0 interval) using 0.1 N HCl and 0.1 N NaOH. The suspension was centrifuged at 3000×*g* for 10 min at 15°C. Following centrifugation, PC in the supernatant was determined by the Lowry method (Lowry et al., 1951). The protein solubility (%) of PG was calculated using the following Equation (1):

$$\text{Protein solubility}\% = \frac{\text{Protein content of supernatant (mg/ml)} \times 50}{\text{Weight of sample (mg)} \times (\text{protein content of sample}(\%)/100)} \times 100 \quad (1)$$

2.5 | Zeta (ζ) potential

The ζ-potential was measured as a function of pH (2.0–13.0, with a 1.0 interval) using a Nano-ZS (Zetasizer NanoZS90, Malvern Instruments). Briefly, 1 g of PG was prepared with distilled water and its pH was adjusted to the desired value using 0.1 N HCl and/or 0.1 N NaOH solutions. The ζ-potential was determined by measuring the direction and velocity of the droplets moving in the electric field applied. The Smoluchowski mathematical model was used by a software (Maplesoft) 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.

2.6 | Ultrasound-assisted enzymatic protein extraction

In the extraction of proteins from *G. dura*, the combinations of ultrasound pretreatment and addition of a carbohydrase enzyme were performed according to the methods of Naseri et al. (2020) and Mæhre et al. (2016) with some modifications. Briefly, 0.5 g of PG was mixed with 50 ml of citrate buffer solution (0.1 N, pH 4.5) and sonicated at a constant frequency of 53 kHz and 65% amplitude during the experimental design periods using an ultrasound homogenizer (Sonopuls HD 2200, Bandelin Electronic GmbH & Co. KG). After sonication, the hemicellulase enzyme was added to the samples and kept in a shaking water bath (N-Biotek-303, Biotek Co., Ltd.) at 55×*g* and 35°C during the extraction time according to the experimental design (Table 1). At the end of extraction, it was kept in the water bath at 85°C for 10 min for enzyme inactivation. Then, the pH of the mixture was adjusted to the pH value where protein solubility of *G. dura* was the highest. Afterward, the samples were subjected to the second extraction by keeping them in the shaking water bath at 35°C for a certain period. Finally, the samples were centrifuged at 3000×*g* for 15 min at 4°C, and the supernatant (or protein extracts from PG) was taken and kept in the dark at −20°C until further analysis.

2.7 | Determination of protein content and extraction efficiency

The protein content of protein extracts from PG (PEPG) was determined using the modified Lowry method (TCA-Lowry) which includes the precipitation of the proteins from the samples with TCA to remove potential interfering substances (Moein et al., 2015). First, 1 ml of PEPG was added to 3 ml of 25% TCA solution and kept in the shaking

TABLE 1 Box–Behnken experimental design with natural and coded extraction conditions and experimentally obtained values of all investigated responses

	Independent variables						Responses			
Run	A: ultrasonic applying time (s)		B: enzyme/substrate ratio		C: extraction time (h)		PC (mg protein/g extract, dw)	TPC (mg GAE/g extract, dw)	AOA_{CUPRAC} (mg TE/g extract, dw)	AOA_{ABTS} (mg TE/g extract, dw)
1	30	−1	0.5	−1	24	0	3.46	19.24	19.43	284.53
2	30	−1	1.5	0	20	−1	84.83	41.06	34.52	398.29
3	165	0	2.5	1	20	−1	163.54	63.70	59.43	417.48
4	300	1	2.5	1	24	0	212.57	57.96	37.45	455.88
5	300	1	1.5	0	28	1	53.31	62.72	49.38	397.58
6	165	0	2.5	1	28	1	110.06	69.45	56.70	478.63
7	165	0	1.5	0	24	0	107.72	40.21	29.35	401.84
8	165	0	1.5	0	24	0	102.16	41.24	31.62	583.85
9	300	1	0.5	−1	24	0	0.51	16.69	11.38	479.34
10	30	−1	1.5	0	28	1	82.07	35.44	40.01	414.64
11	300	1	1.5	0	20	−1	51.73	47.34	72.86	695.47
12	165	0	1.5	0	24	0	101.20	102.57	50.00	434.35
13	165	0	0.5	−1	20	−1	4.86	19.53	25.12	475.47
14	165	0	1.5	0	24	0	128.30	37.10	33.97	512.25
15	30	−1	2.5	1	24	0	160.91	87.73	116.94	432.91
16	165	0	1.5	0	24	0	92.13	40.20	66.62	538.94
17	165	0	0.5	−1	28	1	13.69	20.08	30.41	406.94

Abbreviations: ABTS, 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt; AOA, antioxidant activity; CUPRAC, cupric reducing antioxidant capacity; PC, protein content; TPC, total phenolic content.

water bath at 55×*g* for 30 min at 4°C. Afterward, the supernatant was removed by centrifugation at 3000×*g* for 20 min. The process was repeated sequentially by adding 10% TCA and 5% TCA solutions to the pellets. Finally, 2 ml of 0.1 N NaOH solution was added to the remaining precipitate and the protein content of the extracts was measured spectrophotometrically according to the method of Lowry et al. (1951). Bovine serum albumin was used as the standard protein and protein content was expressed as mg/g in dry weight (dw).

The extraction efficiency was calculated using the following Equation (2)

$$\text{Extraction efficiency}\,\% = \frac{(\text{The protein content of the extract after extraction}) \times (\text{The content of extract after extraction})}{(\text{The content of macroalgal protein before extraction}) \times (\text{The content of macroalgae before extraction})} \times 100 \quad (2)$$

2.8 | Total phenolic content

The TPC of PEPG was determined according to Folin–Ciocalteu's method (Toor & Savage, 2006). Briefly, 200 µl of PEPG, 1.5 ml of Folin–Ciocalteu's reagent:H_2O (1:10, v/v), and 1.2 ml of aqueous 7.5% Na_2CO_3 were mixed and allowed to stand at room temperature in the dark for 90 min. The absorbance was read at 765 nm using an ultraviolet–visible spectrophotometer (Scilogex Sci-UV1000 Spectrophotometer, Scilogex). Gallic acid was used as standard and TPC was calculated using a linear equation from the calibrated curve. The results were expressed as mg gallic acid equivalent (GAE)/g dw.

2.9 | Antioxidant activity

2.9.1 | The cupric reducing antioxidant capacity method

The cupric reducing antioxidant capacity (CUPRAC) assay was performed according to the method of Apak et al. (2005). Briefly,100 µl of PEPG was mixed with 1 ml each of 10^{-2} N copper (II) chloride solution, 7.5×10^{-3} N neocuproine solution, ammonium acetate buffer solution (pH 7.0), and distilled water. After 30 min of incubation at room temperature, the absorbance of the mixture was measured at 450 nm using the UV spectrophotometer. Results were expressed as milligrams mg Trolox equivalent (TE)/g dw.

2.9.2 | 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt method

2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) assay was performed according to Miller and Rice-Evans (1997). The ABTS stock solution was prepared with ABTS and

potassium persulfate solutions and kept at room temperature in the dark overnight. The ABTS stock solution was diluted in 50 mM potassium phosphate buffer (pH 8.0) to an absorbance of 0.90 (±0.05) at 734 nm to prepare the ABTS-working solution. Then, 100 µl of PEPG was mixed with 1 µl of ABTS-working solution and the absorbance was measured at 734 nm exactly 1 min after initial mixing. Results were expressed as mg TE/g dw.

2.10 | Techno-functional properties

2.10.1 | Water absorption capacity

Water absorption capacity (WAC) was performed using the method of Kumar et al. (2014). Briefly, 0.1 g of PEPG 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 for 30 min and centrifuged at 3000×*g* for 20 min. The supernatant was removed and the centrifuge tube containing sediment was weighed. The WAC was calculated using the following Equation (3):

$$\mathrm{WAC}(\%) = \frac{W_2 - W_1}{W_0} \times 100 \quad (3)$$

where W_0 is the weight of PEPG (g), W_1 is the weight of the tube containing PEPG (g), W_2 is the weight of the tube after decantation of water (g).

2.10.2 | Oil absorption capacity

Oil absorption capacity (OAC) was employed according to the method of Kumar et al. (2014). Briefly, 1.0 g of PEPG obtained under optimum extraction conditions was dispersed in 5 ml of sunflower oil and centrifuged at 3000×*g* for 20 min, the supernatant was discharged and the tubes were weighed. The OAC was calculated using the following Equation (4):

$$\mathrm{OAC}(\%) = \frac{O_2 - O_1}{O_0} \times 100 \quad (4)$$

where O_0 is the weight of PEPG (g), O_1 is the weight of the tube containing PEPG (g) and O_2 weight of the tube after decantation of oil (g).

2.10.3 | Emulsifying activity and stability

Emulsifying activity (EA) and emulsifying stability (ES) were determined using the method of Tan et al. (2014). Briefly, 0.1 g of PEPG obtained under optimum extraction conditions was diluted with 10 ml of distilled water and homogenized for 2 min at room temperature using a hand-held homogenizer (MT-30K MIULAB Handheld Homogenizer, Hangzhou Miu Instruments Co. Ltd.). After the homogenization, 10 ml of olive oil was added to the mixture and homogenized again under the same conditions. At the end of the time, the mixture was centrifuged at 1200×*g* for 5 min. The height of the emulsion layer was recorded and the emulsion activity of the emulsion sample was calculated using the following Equation (5):

$$\mathrm{EA}(\%) = \frac{\text{Height of emulsified layer}}{\text{Height of contents of tube}} \times 100 \quad (5)$$

For the determination of ES, the samples were heated at 80°C for 30 min, then centrifuged at 1200×*g* for 5 min. The ES of the samples was calculated using the following Equation (6):

$$\mathrm{ES}(\%) = \frac{\text{Height of remaining emulsified layer}}{\text{Height of original emulsified layer}} \times 100 \quad (6)$$

2.10.4 | Foaming capacity and stability

Foaming capacity (FC) and foaming stability (FS) assays of PEPG extracted under optimum extraction conditions were carried out according to the method of Jarpa-Parra et al. (2014). Briefly, 0.02 mg of PEPG extracted under optimum extraction conditions was added to 20 ml of distilled water and whipped for 2 min using the handheld homogenizer. The FC was calculated using the following Equation (7):

$$\mathrm{FC}(\%) = \frac{\text{Volume after whipping(ml)} - \text{Volume before whipping(ml)}}{\text{Volume before whipping(ml)}} \times 100 \quad (7)$$

After the homogenization, the mixture was held at room temperature for 30 min. The FS was calculated using the following Equation (8):

$$\mathrm{FS}(\%) = \frac{\text{Volume after standing(ml)} - \text{Volume before whipping(ml)}}{\text{Volume before whipping(ml)}} \times 100 \quad (8)$$

2.10.5 | Fourier transform infrared spectroscopy

Organic groups in PEPG obtained under optimum extraction conditions were characterized using the FT-IR spectroscopy technique (Bruker Tensor II FTIR spectrometer equipped with the ATR diamond module (Bruker Optics). All the spectra were an average of 18 scans from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}.

2.11 | Experimental design and statistical analysis

Response surface methodology based on Box–Behnken Design (BBD) was used for the optimization of extraction conditions. The independent variables were ultrasonic applying time (30–300 s), E/S ratio (0.5–2.5), and extraction time (20–28 h; Table 1). The experimental design consists of 17 experimental runs, including 12 factorial points and five replicates at central points.

The response variables were fitted to a second-order polynomial model to obtain the regression coefficients (β). The generalized

second-order polynomial model was used in the response surface analysis using the following Equation (9):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \epsilon\pi r^2 \quad (9)$$

The regression coefficient is defined as β_0 for constant, β_i for linear, β_{ii} for quadratic, and β_{ij} for interaction effect term. The analysis of variance (ANOVA) was performed to determine regression coefficients and statistical significance, as well as to evaluate model appropriateness. Statistical analysis was performed using Design Expert 7.1 software (Stat-Ease, Inc.). The results were statistically tested at the significance level of $p = 0.05$. The coefficient of determination (R^2) and model weakness were used to assess the model's suitability. A mathematical model was obtained to describe the influence of the single process parameter and/or interaction of multiple parameters on each investigated response.

Experimental data were expressed as mean ± standard deviation (SD). Statistical analysis was performed on data using a statistical package (Minitab, Version 17, Minitab Inc.). The differences between mean values were compared using the Tukey test. Differences at $p \leq 0.05$ were considered to be significant.

3 | RESULTS AND DISCUSSION

3.1 | Proximate composition and profiles of fatty acid, carbohydrates, and minerals of PG

The proximate composition of PG was given in Table 2. The protein content of PG was determined as 11.97 ± 0.05%. Similar to in the present study, the protein content of some *Gracilaria* species ranged from 5.6% to 30.2% (Chan & Matanjun, 2017; Gressler et al., 2010; Rodrigues et al., 2015; Valente et al., 2006). Soluble carbohydrate and crude fiber contents of PG were 46.78 ± 5.09% and 16.77 ± 1.14%, respectively (Table 2). Similarly, total carbohydrate content was found to be 46.6% for *G. gracilis*, 43.2% for *G. turuturu* by Rodrigues et al. (2015), and 41.52% for *G. changii* by Chan and Matanjun (2017). According to Xu et al. (2015), carbohydrate content of *Gracilaria* species changed between 15% and 63%. On the contrary, Tabarsa et al. (2012) reported that the crude fiber of *G. salicornia* was 10.4 ± 0.89%. In the present study, the soluble carbohydrate profile of PG was determined as myo-inositol (1379.35 ± 214.57 mg/kg dw) and fructose (216.84 ± 66 mg/kg dw). However, glucose, saccharose, and mannitol were not detected in the present study (Table 2).

In the study, the lipid content of PG (0.32 ± 0.06%, Table 2) was found to be similar to *G. gracilis* (0.60%) and *G. turuturu* (2.2%; Rodrigues et al., 2015). On the contrary, the lipid content of some *Gracilaria* species varied from 0.7% to 2.8% (Gressler et al., 2010; McDermid & Stuercke, 2003). According to literature, macroalgae had low lipid content (less than 4%; Manivannan et al., 2008; McDermid & Stuercke, 2003). In the present study, saturated fatty acids in PG were capric acid (decanoic acid) C10:0 (2.77 ± 0.22%), lauric acid (dodecanoic acid) C12:0 (0.84 ± 0.00%), myristic acid C14:0 (11.34 ± 0.29%), palmitic acid C16:0 (69.73 ± 0.53%), stearic acid C18:0 (3.88 ± 0.44%; Table 2). The major fatty acid in PG was palmitic acid C16:0 (69.73 ± 0.53%). According to literature, palmitic acid is the most abundant fatty acid in *Gracilaria* species (Khotimchenko et al., 2002; Wen et al., 2006). Similarly, Gressler et al. (2010) stated that palmitic acid was found to be the major acid in *G. domingensis* (65.4%) and *G. birdiae* (56.9%). Moreover, Rodrigues et al. (2015)

TABLE 2 Proximate composition of *Gracilaria dura* in wet and dry weight. Data were given as mean values ± standard deviation ($n = 3$)

	Wet weight, %	Dry weight, %
Carbohydrate	43.28 ± 4.71	46.78 ± 5.09
Protein	11.07 ± 0.05	11.97 ± 0.05
Lipid	0.29 ± 0.06	0.32 ± 0.06
Moisture	7.50 ± 0.50	
Crude fiber	15.52 ± 1.06	16.77 ± 1.14
Total ash	22.43 ± 0.50	24.15 ± 0.50
Fatty acid profile (%)		
C10	2.56 ± 0.20	2.77 ± 0.22
C12	0.78 ± 0.00	0.84 ± 0.00
C14	10.49 ± 0.27	11.34 ± 0.29
C15-1	0.98 ± 0.15	1.06 ± 0.16
C16	64.05 ± 0.49	69.73 ± 0.53
C16-1	5.48 ± 0.47	5.92 ± 0.51
C18	3.59 ± 0.415	3.88 ± 0.44
C18-1 cis	9.66 ± 0.08	10.44 ± 0.08
C18-2 cis	1.12 ± 0.36	1.21 ± 0.38
C18-3n6	1.52 ± 0.00	1.64 ± 0.00
Soluble carbohydrate profile (mg/kg)		
Myo-inositol	1275.90 ± 198.48	1379.35 ± 214.57
Mannitol	n.d.	n.d.
Glucose	n.d.	n.d.
Fructose	200.58 ± 61.05	216.84 ± 66
Saccharose	n.d.	n.d.
Mineral profile (μg/kg)		
Al	588.92 ± 7.63	636.67 ± 8.24
Mn	102.79 ± 0.99	111.12 ± 1.07
Fe	740.35 ± 8.78	800.37 ± 9.49
Ni	3.39 ± 0.05	3.66 ± 0.05
Cu	5.04 ± 0.14	5.45 ± 0.15
Zn	19.4 ± 0.34	20.97 ± 0.36
Se	0.91 ± 0.01	0.98 ± 0.01
Ag	4.18 ± 0.07	4.52 ± 0.07
Cd	0.04 ± 0.00	0.04 ± 0.00
Pb	1.04 ± 0.02	1.12 ± 0.02
Mg	2594.4 ± 30.06	2804.75 ± 32.50
K	7193.8 ± 67.90	7777.08 ± 73.40

Abbreviation: n.d., not detected.

reported that the most abundant fatty acid in *G. gracilis* (52.54%) and *G. turuturu* (35.88%) was palmitic acid. Unsaturated fatty acids in PG were pentadecenoic acid C15:1 (1.06 ± 0.16%), palmitoleic acid C16:1 (5.92 ± 0.51%), oleic acid C18:1 cis (ω-9, 10.44 ± 0.08%), linoleic acid C18:2 cis (ω-6, 1.21 ± 0.38%), γ-linolenic acid C18:3n6 (1.64 ± 0.00%; Table 2). Similarly, *Gracilaria* species had unsaturated fatty acids such as C16:1 (0.01 ± 0.0%), C18:1 (0.05 ± 0.0%), and C18:2 (0.01 ± 0.0%; Gressler et al., 2010).

The total ash content of PG was calculated as 24.15% in dw. In the literature, the ash content of *Gracilaria* species ranged from 22.7% to 53.4% (Gressler et al., 2010; Norziah & Ching, 2000). In addition to, the mineral profile of PG composed of Al (636.67 ± 8.24 μg/kg), Mn (111.12 ± 1.07 μg/kg), Fe (800.37 ± 9.49 μg/kg), Ni (3.66 ± 0.05 μg/kg), Cu (5.45 ± 0.15 μg/kg), Zn (20.97 ± 0.36 μg/kg), Se (0.98 ± 0.01 μg/kg), Ag (4.52 ± 0.07 μg/kg), Cd (0.04 ± 0.00 μg/kg), Pb (1.12 ± 0.02 μg/kg), Mg (2804.75 ± 32.50 μg/kg), K (7777.08 ± 73.40 μg/kg; Table 2). Tabarsa et al. (2012) indicated that potassium was the most abundant essential element in red macroalgae and was found to be 11,380.06 ± 73.45 μg/kg dw in *G. salicornia*. In the present study, the obtained data about the chemical composition of *G. dura* is generally closed to previous studies as explained in detail above. However, the chemical composition of macroalgae may change depending on the species, seasons, and habitat (Bilgin & Ertan, 2002).

3.2 | Protein solubility and zeta potential

In this study, the effect of pH on net surface charges (zeta potential) and protein solubility of PG was investigated based on the zeta potential measurements (Figure 1). As seen in Figure 1, the zeta potential was found to range from −8.41 ± 0.71 mV at pH 6.0 to −20.73 ± 0.38 mV at pH 12.0. All zeta potential values of PG were found to be as negative because of the negative charges of polysaccharides in the extracts (Evans et al., 2013). In contrast, 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 and these negative groups can interact with other positive groups. However, the negative charges can be much more numerous and affect the surface charge (Rosenhahn et al., 2009).

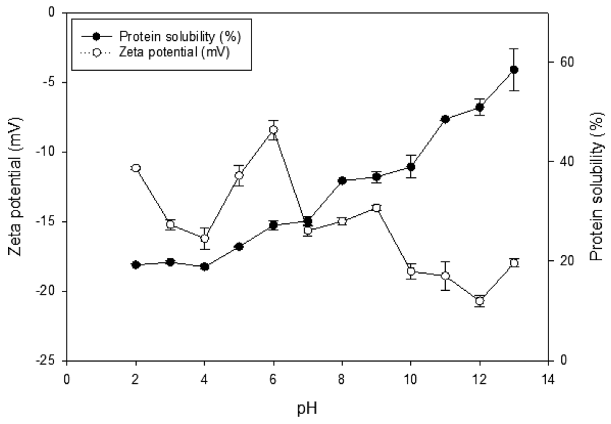

FIGURE 1 Effect of pH on zeta potential (mV) and protein solubility of protein extract from *Gracilaria dura*.

Protein solubility is correlated with surface charge and pH value. The minimum solubility (18.87 ± 0.37%) of *Gracilaria dura* proteins occurred at pH 4.0. Similarly, Böcker et al. (2021) obtained the lowest nitrogen solubility of *Arthrospira platensis* at pH 3.5. In the present work, the maximum solubility of *Gracilaria dura* was found as 58.53 ± 4.26% at pH 13.0. Likewise, Guil-Guerrero et al. (2004) reported that the highest nitrogen solubility for *Phaeodactylum tricornutum* was found to be 75% at pH 12. On the contrary, it was 20% at pH 12 for *Porphyridium cruentum* due to the presence of a cell wall.

3.3 | Model fitting

The PC, TPC, and AOA were determined as functions of linear, quadratic, and interaction terms of the independent variables including the ratio of E/S, ultrasonic applying time, and extraction time using BBD (Table 1). Analysis of variance and coefficients of the model (R^2) for each dependent variable are indicated in Table 3.

The R^2 values were 0.93, 0.60, 0.61, and 0.74 for PC, TPC, AOA_{CUPRAC}, and AOA_{ABTS}, respectively (Table 3). Except for PC (>0.80), TPC, AOA_{CUPRAC}, and AOA_{ABTS} have low R^2 for the models. The high values of R^2 indicated the fit of the model (Moorthy et al., 2015). The variation coefficient (CV) of the model can be low as an indication of good reproducibility of the investigated systems. However, except AOA_{ABTS} (CV = 15.15%); PC (CV = 28.04%), TPC (CV = 49.19%), and AOA_{CUPRAC} (CV = 52.01%) showed high variation in their mean values. The lack of fit was not significant for PC, TPC, AOA_{CUPRAC}, and AOA_{ABTS} ($p > 0.05$). These results demonstrated that the model for protein content ($p = 0.0028$) can be used to optimize the extraction parameters for the extraction of proteins from *Gracilaria dura*.

The linear effect of E/S on PC, TPC, and AOA_{CUPRAC} was statistically significant ($p < 0.05$). Especially, its effect on PC was extremely significant ($p < 0.0001$; Table 3). Conversely, ultrasonic applying time and extraction time had no significant effects on PC, TPC, AOA_{CUPRAC}, and AOA_{ABTS}. However, the quadratic effect of extraction time on PC was significant ($p < 0.05$).

3.4 | Protein content of the extracts

As seen in Table 1, the highest PC was obtained as 212.57 mg/g under the applied extraction conditions (ultrasonic applying time of 165 s, E/S of 2.5, and extraction time of 20 h). The interaction effect of E/S and ultrasonic applying time at constant extraction time (24 h), is shown in Figure 2a. The graph plot revealed that protein content of PEPG increased under the experimental conditions of ultrasonic applying time of upper ~98 to 300 s and E/S of ~2.20 (Figure 2a). The linear effect of E/S on PC was statistically significant

TABLE 3 Analysis of variance (ANOVA) of the fitted second-order polynomial model

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value
PC					
Model	55070.80	9	6118.98	10.36	0.0028*
Linear					
β_1	21.66	1	21.66	0.037	0.8535
β_2	48761.86	1	48761.86	82.59	<0.0001*
β_3	262.60	1	262.60	0.44	0.5262
Quadratic					
β_{11}	303.94	1	303.94	0.51	0.4963
β_{22}	49.96	1	49.96	0.085	0.7796
β_{33}	3743.94	1	3743.94	6.34	0.0399*
Interaction					
β_{12}	745.80	1	745.80	1.26	0.2981
β_{13}	4.71	1	4.71	7.975E−003	0.9313
β_{23}	970.88	1	970.88	1.64	0.2406
Residual	4133.08	7	590.44	—	—
Lack of fit	3402.82	3	1134.27	6.21	0.0550
Pure error	730.26	4	182.56	—	—
Cor total	59203.88	16	—	—	—
$R^2 = 0.93$; CV (%) = 28.04					
TPC					
Model	5708.21	9	634.25	1.18	0.4212
Linear					
β_1	0.19	1	0.19	3.5639	0.9855
β_2	5166.68	1	5166.68	65	0.0172*
β_3	32.25	1	32.25	0.060	0.8132
Quadratic					
β_{11}	12.25	1	12.25	0.0230	0.8841
β_{22}	111.93	1	111.93	21	0.6614
β_{33}	64.74	1	64.74	0.12	0.7383
Interaction					
β_{12}	185.25	1	185.25	0.35	0.5749
β_{13}	110.26	1	110.26	0.21	0.6638
β_{23}	6.75	1	6.75	0.013	0.9138
Residual	3749.35	7	535.62	—	—
Lack of fit	576.90	3	192.30	0.24	0.8630
Pure error	3172.46	4	793.11	—	—
Cor total	9457.56	16	—	—	—
$R^2 = 0.60$; CV (%) = 49.19					
AOA_{CUPRAC}					
Model	6101.17	9	677.91	1.24	0.3985
Linear					
β_1	198.32	1	198.32	0.36	0.5665
β_2	4240.62	1	4240.62	7.74	0.0272*
β_3	29.77	1	29.77	0.054	0.8224

TABLE 3 (Continued)

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value
Quadratic					
β_{11}	110.87	1	110.87	0.20	0.6665
β_{22}	5.48	1	5.48	9.997E−003	0.9232
β_{33}	12.86	1	12.86	0.023	0.8826
Interaction					
β_{12}	1276.09	1	1276.09	2.33	0.1709
β_{13}	209.80	1	209.80	0.38	0.5557
β_{23}	16.07	1	16.07	0.029	0.8689
Residual	3836.85	7	548.12	—	—
Lack of fit	2834.79	3	944.93	3.77	0.1161
Pure error	1002.06	4	250.52	—	—
Cor total	9938.02	16			
$R^2 = 0.61$; CV (%) = 52.01					
AOA_{ABTS}					
Model	96761.92	9	10751.32	2.22	0.1529
Linear					
β_1	30987.54	1	30987.54	6.40	0.0393*
β_2	2401.84	1	2401.84	0.50	0.5041*
β_3	10434.58	1	10434.58	2.15	0.1856
Quadratic					
β_{11}	2550.03	1	2550.03	0.53	0.4916
β_{22}	13428.48	1	13428.48	2.77	0.1398
β_{33}	197.95	1	197.95	0.041	0.8455
Interaction					
β_{12}	7382.36	1	7382.36	1.52	0.2568
β_{13}	24688.33	1	24688.33	5.10	0.0585
β_{23}	4203.26	1	4203.26	0.87	0.3826
Residual	33904.47	7	4843.50	—	—
Lack of fit	11428.12	3	3809.37	0.68	0.6097
Pure error	22476.35	4	5619.09	—	—
Cor total	1.307E+005	16			
$R^2 = 0.74$; CV (%) = 15.15					

Abbreviations: β_1, ultrasonic applying time (s); β_2, enzyme/substrate ratio; β_3, extraction time (h); ABTS, 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt; AOA, antioxidant activity; CUPRAC, cupric reducing antioxidant capacity; PC, protein content; TPC, total phenolic content.
*Significant at $p \leq 0.05$.

($p < 0.05$; Table 1). This might be because the algal cell wall was destroyed by the used enzyme, thus more protein was released into the solvent medium. Similarly, Joubert and Fleurence (2008) investigated the effect of xylanase and cellulase enzymes and also enzyme concentration on the protein content of *Palmaria palmata* and reported that protein content increased as the amount of enzyme increased. Likewise, Harnedy and FitzGerald (2013) reported that the utilization of polysaccharidase to break down cell wall caused an increase in the extraction efficiency of proteins from macroalgae. Furthermore, Suwal et al. (2019) reported that the cellulase enzyme used in extraction increased the protein content by 17% in *Solieria chordalis*. According to Table 3, the quadratic effect of the extraction time was significant ($p < 0.05$). Similar results (73.6 ± 1.2%) were obtained where the extraction time was increased from 2 to 18 h for ultrasound-assisted extraction of *Chlorella vulgaris* and the longer extraction time increased the extraction yield (Hildebrand et al., 2020).

3.5 | Total phenolic content and antioxidant activity

The TPC of PEPG ranged from 16.69 mg GAE/g to 102.57 mg GAE/g under the extraction conditions given in Table 1. Similarly, Nursid et al. (2020) obtained TPC as 23.37 mg GAE/g for *Gracillaria*

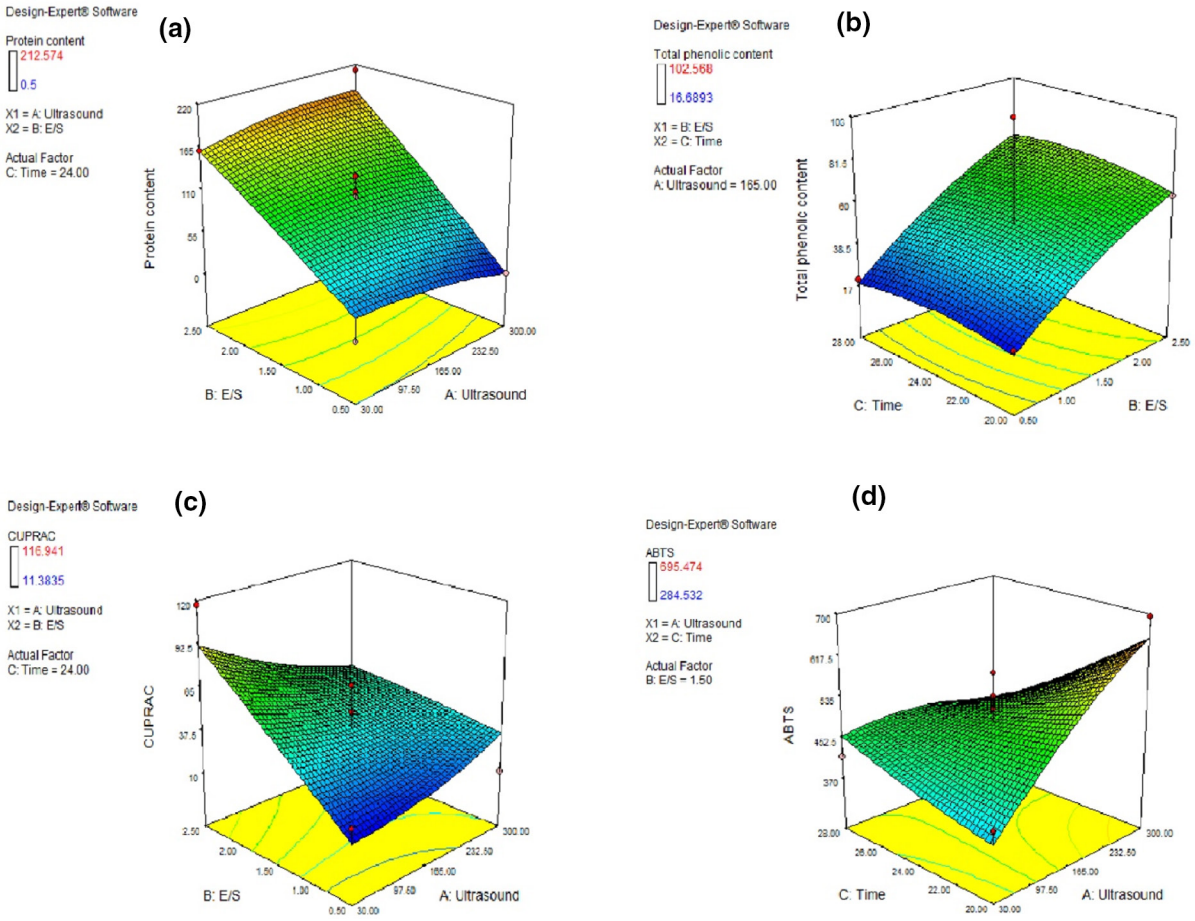

FIGURE 2 The interaction effects between ultrasonic applying time and enzyme/substrate ratio on the protein content of the protein extracts at constant extraction time of 24 h (a), between extraction time and enzyme/substrate ratio on total phenolic content at constant ultrasonic applying time of 165 s (b), between ultrasonic applying time and enzyme/substrate ratio on antioxidant activity by cupric reducing antioxidant capacity (CUPRAC) method at constant extraction time of 24 h (c) and between ultrasonic applying time and extraction time on antioxidant activity by 2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) method of the extracts at constant enzyme/substrate ratio of 1.50 (d).

salicornia, 24.97 mg GAE/g for *Laurencia sp.*, and 24.38 mg GAE/g for *Gelidium latifolium*. In contrast, Neto et al. (2018) determined the TPC of *Gracilaria* sp. as 5.9 ± 0.03 mg GAE/g. Additionally, Yildiz et al. (2011) determined TPC of *Gracilaria bursa-pastoris* as 0.35 mg GAE/g and Zhang et al. (2007) stated that the TPC of *Gracilaria gracilis* as 0.10 mg GAE/g.

The AOA_{CUPRAC} and AOA_{ABTS} of PEPG ranged from 11.15 mg TE/g to 102.48 mg TE/g and from 284.53 mg TE/g to 695.47 mg TE/g under the extraction conditions given in Table 1. Yuan et al. (2018) reported the highest AOA_{ABTS} for *Lessonia nigrecens* as 0.95 ± 0.01 mg TE/g dw. The AOA_{FRAP} (AOA-ferric reducing antioxidant power) of *Gracilaria gracilis* and *Laminaria digitata* was found to be 6.26 mg TE/g and 3.99 mg TE/g, respectively (Heffernan et al., 2015). Kumar et al. (2020) obtained AOA_{FRAP} as 8.21 mg TE/g for *Sargassum wightii*, 6.90 mg TE/g for *Ulva rigida*, and 1.06 mg TE/g for *Gracilaria edulis*. Nursid et al. (2020) reported that these differences in polyphenol content and antioxidant activity may be caused by following the season in which macroalgae were collected, harvest time, geographical location, and algae species.

The linear effect of E/S on TPC and AOA_{CUPRAC} of PEPG and the linear effect of the ultrasonic applying time on AOA_{ABTS} were statistically significant (Table 3, $p < 0.05$). Likewise, Kadam et al. (2015) reported that ultrasound-assisted extraction increased the extraction of phenolic compounds. Similar to TPC, the AOA of PEPG increased with an increase in enzyme amount since polyphenolics have high antioxidant activity ($p < 0.05$; Ozdal et al., 2013). Like PC, the effect of E/S was found to be high on TPC ($p < 0.05$), because phenolic compounds are covalently bound to proteins (Acosta-Estrada et al., 2014). Wijesinghe and Jeon (2012) stated that enzyme-assisted degradation of cell wall polysaccharides increases the release of phenolic compounds. Moreover, the total phenolic content of PEPG increased under the experimental conditions of E/S of ~1.5 (Figure 2b). On the contrary, as seen in Figure 2c, AOA_{CUPRAC} of PEPG increased at E/S of ~2.2 to 2.5 and ultrasonic applying time of 30–60 s with constant extraction time (24 h). In addition, AOA_{ABTS} of PEPG was the highest when ultrasonic applying time of ~235 to 300 s, and extraction time of ~20 to 22 h at constant E/S (1.5) were applied (Figure 2d).

3.6 | Extraction yield

In the present study, the extraction yield of PEPG extracted under optimum extraction conditions (ultrasonic applying time of 257.57 s, E/S of 2.5, and extraction time of 22.61 h) was found as 95 ± 4.95%. Similarly, Naseri et al. (2020) obtained the highest protein extraction yield for *Palmaria palmata* as >80% with alcalase enzyme. In the study of Kadam et al. (2017), protein extraction yield of *Ascophyllum nodosum* ranged from 7.71% to 59.76 depending on applied different extraction processes. Suwal et al. (2019) reported that using a cell wall degrading enzyme enhanced extraction yield from 9% to 37% for *Palmaria palmata*. Furthermore, the extraction yield was found to be 74.6% for *Macrocystis pyrifera* and 36.1% for *Chondracanthus chamissoi* using cellulase enzyme in the study conducted by Vásquez et al. (2019). On the contrary, Barbarino and Lourenço (2005) reported that the chemical composition of the species, as well as its morphological and structural properties, have a direct impact on their extraction yield.

3.7 | Optimization and verification

Optimization procedures were carried out to predict the optimum level of independent variables to obtain maximum values for PC, TPC, AOA_{CUPRAC}, and AOA_{ABTS}. Under the optimum conditions (ultrasonic applying time of 257.57 s, E/S of 2.5, and extraction time of 22.61 h), the predicted PC, TPC, AOA_{CUPRAC}, and AOA_{ABTS} values were 188.71 mg/g dw, 64.33 mg GAE/g dw, 54.24 mg TE/g dw and 477.44 mg TE/g dw, respectively in a "desirability" of 0.89. The predicted and mean of experimental values for PC (188.71 mg protein/g dw and 189.59 ± 22.80 mg protein/g dw), TPC (64.33 mg GAE/g dw and 64.23 ± 3.77 mg GAE/g dw), AOA_{CUPRAC} (54.24 mg TE/g dw and 55.66 ± 0.91 mg TE/g dw), and AOA_{ABTS} (477.44 mg TE/g dw and 478.50 mg TE/g dw) were not statistically different at the 5% significance level.

3.8 | Techno-functional properties

3.8.1 | Water and oil absorption capacity

The techno-functional properties of PEPG obtained under optimum extraction conditions were determined. The WAC of a protein is a critical property in viscous foods (soup, dough, bakery product) to maintain mouthfeel, thickening, and viscosity (Kandasamy et al., 2012). According to the results, the WAC of PEPG was calculated as 195 ± 0.08%. Similarly, Kandasamy et al. (2012) found that the WAC of *Enteromorpha compressa*, *Enteromorpha tubulosa*, and *Enteromorpha linza* were 153 ± 0.07%, 132 ± 0.11%, and 122 ± 0.06%, respectively. In contrast, Kumar et al. (2014) stated that WAC of *Kappaphycus alvarezii* was 222 ± 0.04%. Benjama and Masniyom (2012) reported that the WAC of *Gracilaria fisheri* and *Gracilaria tenuistipita* were 553 ± 0.02% and 897 ± 1.73%, respectively. Moreover, Yücetepe et al. (2019) reported that the WAC values of *Spirulina platensis* ranged from 335 to 512%. Conversely, the WAC of protein extracts from *Chlorella vulgaris* was determined as 12.19 ± 3.84% in the study of Yucetepe (2022). On the contrary, the WAC of *G. dura* protein isolate was lower than those of soy protein isolate (447 ± 0.00%) and red kidney (225 ± 0.13%), while it was higher than those of some plant seed proteins such as chickpea (119 ± 0.01%) and lentil (133 ± 0.02%; Table 4; Du et al., 2014; Kinsella, 1979; Siddiq et al., 2010).

The OAC of proteins is an important functional property, especially for meat, sausage, and mayonnaise (Chandi & Sogi, 2007). The OAC values of *G. dura* proteins and selected foods rich in protein are given in Table 4. The OAC of PEPG was determined as 568 ± 0.04%. In the present study, the OAC of PEPG was higher than those of some macroalgae. For instance, OAC of *K. alvarezii* was found to be 129 ± 0.20% by Kumar et al. (2014). Kandasamy et al. (2012) investigated the OAC of several macroalgae including *E. compressa* (134 ± 0.10%), *E. tubulosa* (108 ± 0.04%), *E. linza* (105 ± 0.07%). Benjama and Masniyom (2012) reported that OAC of *G. fisheri* and *G. tenuistipita* were 179 ± 0.07% and 223 ± 0.15%, respectively. Furthermore, the OAC of PEPG was higher than soy protein isolate (36 ± 0.2%), whey protein isolate (19 ± 0.1%), and egg protein (21 ± 0.0%), as seen in Table 4 (Lam et al., 2017). These differences in values of OAC and WAC resulted from amino acid composition and protein conformation of protein, and extraction methods/parameters. In the present work, *G. dura* with acceptable WAC and OAC values can be considered a suitable candidate for food applications such as moisture holding or texture enhancer.

3.8.2 | Foaming and emulsifying properties

In the present study, the FC of PEPG was 12.5 ± 0.0%. This value was lower than the FC of *E. compressa* (55.0 ± 2.6%), *E. tubulosa* (31.9 ± 2.7%), and *E. linza* (33.3 ± 5.7%; Kandasamy et al., 2012) and *K. alvarezii* (38 ± 2.0% at pH 6.0, 53.33 ± 2.31% at pH 4.0; Kumar et al., 2014). Similarly, the FC of PEPG was lower than those of some plant proteins such as soybean protein (65.7 ± 0.5%) and whey protein (132%; Ijarotimi et al., 2018; Jambrak et al., 2008). The FC and FS values of *G. dura* proteins and selected foods rich in protein are given in Table 4. Furthermore, the proteins from *G. dura* did not exhibit foaming stability. Conversely, Kumar et al. (2014) found to be as 16.7 ± 1.5%, 37.5 ± 2.0%, 4.4 ± 2.0, 45.33 ± 1.15% for FS of *E. tubulosa*, *E. compressa*, *E. linza*, *K. alvarezii*, respectively. Extraction procedure, macroalgal genotype, foam formation process, and environmental conditions can affect the FC and FS of proteins (Makri & Doxastakis, 2006). Moreover, the foaming capacity of proteins is affected by solubility, surface charge, and the balance between hydrophilic and hydrophobic amino acids (Gundogan & Karaca, 2020; Zheng et al., 2020).

TABLE 4 Techno-functional properties of *G. dura* proteins and selected foods rich in protein

Sample	WAC (%)	OAC (%)	EA (%)	ES (%)	FC (%)	FS (%)	Reference
Gracilaria dura	195 ± 0.08	568 ± 0.00	44 ± 0.0%	75 ± 2.50%	12.5 ± 0.0%	0 ± 0.0%	
Gracilaria fisheri	553 ± 0.22	179 ± 0.07	–	–	–	–	Benjama and Masniyom (2012)
Gracilaria tenuistipitata	897 ± 1.73	223 ± 0.15	–	–	–	–	Benjama and Masniyom (2012)
Chickpea	119 ± 0.01	110 ± 0.02	61.14 ± 0.61	94.19 ± 1.64	52.00	83.0	Du et al. (2014)
Lentil	133 ± 0.02	93 ± 0.00	65.75 ± 0.11	91.99 ± 4.75	80.00	99.0	Du et al. (2014)
Soy protein isolate	447	154	25.00	–	235.00	–	Kinsella (1979)
Red kidney	2.25 ± 0.13	1.52 ± 0.11	55.00 ± 1.80	52.40 ± 1.80	45.70 ± 1.40	41.20 ± 1.80	Siddiq et al. (2010)
Peanut	1.30	0.90	57.00	–	51.00	96.00	Lam et al. (2017)
Whey protein isolate	–	1.9 ± 0.1	–	89.3 ± 1.2	302.2 ± 13.9	78.7 ± 1.9	Lam et al. (2017)
Pea protein isolate	–	1.5 ± 0.0	–	79.3 ± 1.2	165.6 ± 31.0	56.6 ± 4.5	Lam et al. (2017)
Wheat protein isolate	–	2.6 ± 0.1	–	63.3 ± 1.2	192.2 ± 3.8	9.8 ± 0.9	Lam et al. (2017)
Egg protein isolate	–	2.1 ± 0.0	–	91.3 ± 1.2	234.4 ± 20.1	79.8 ± 4.2	Lam et al. (2017)

The EA is defined as the capacity of the protein to assist in the formation and stabilization of the formed emulsion. The ES, on the contrary, is defined as a protein's ability to stabilize an emulsion without affecting its structure (e.g., coalescence, creaming, aggregation, or precipitation) over a period of time (Boye et al., 2010; Karaca et al., 2011). The EA and ES of PEPG were found to be 44 ± 0.00% and 75 ± 2.50%, respectively. Likewise, Kumar et al. (2014) reported that the emulsification index of *K. alvarezii* protein was approximately 60–62%. The EA and ES of *Spirulina platensis* proteins were found to be 40%–45.98% and 83.33%–100% by Yücetepe et al. (2019). As seen in Table 4, EA of *G. dura* was lower than those of chickpea (61.14%), lentil (65.75%), and red kidney (55.00%), while higher than that of soy protein isolate (25.00%; Du et al., 2014; Kinsella, 1979; Siddiq et al., 2010). Lam et al. (2017) investigated the ES of several pea cultivars including soy protein isolate (94.3%), whey protein isolate (89.3%), wheat protein isolate (63.3%), and egg protein (91.3%; Table 4). The EA and ES properties might vary depending on the hydrophobicity, net surface charge, and solubility (Shevkani et al., 2015).

3.8.3 | FT-IR

The secondary structural composition of proteins is important in terms of their bioactive and functional properties. Their secondary structures can be explained with the FT-IR technique (Kong & Yu, 2007). The FT-IR spectra of PEPG are exhibited in Figure 3. The FT-IR spectrum of PEPG indicated absorption bands at ~1630 cm^{-1} (amide I, C=O stretching as free carboxyl groups) and ~1508 cm^{-1} (amide II, C–N stretching, and N–H bending. Similarly, Kong and Yu (2007) reported the amide II region at wavelengths of 1480–1585 cm^{-1} and Carbonaro et al. (2008) stated the amide I region at the wavelength of 1600–1700 cm^{-1}. Amid I and II bands are the main peaks indicating the presence of proteins in the structure (Withana-Gamage et al., 2011). Amide I band of proteins is a complex structure, which carries multiple components such as α-helix, β-sheet, random ring, or β-turn (Liu et al., 2009). Peaks at the wavelength of ~1287 cm^{-1} showed an amide III band (C–C, C–N, and C–O stretching). de la Rosa-Millán et al. (2018) stated that amide III demonstrates the existence of interactions between protein and other macromolecules such as carbohydrates. The extracts indicated the amide A region (O–H stretching vibration) at the wavelength of ~3242 cm^{-1}. Feyzi et al. (2018) indicated that the amide A region at 3200–3500 cm^{-1} represents an interaction between protein and water molecules. The amide B region (asymmetric stretch vibration of C–H) was assigned a wavelength of ~2851 cm^{-1} and this region indicated the existence of neutral lipids, proteins, and carbohydrates (Withana-Gamage et al., 2011). Peaks at the wavelength of ~1052 cm^{-1} were observed in the FT-IR spectrum and these peaks indicated the existence of carbohydrates in PEPG. According to Pietrzak and Miller (2005), peaks observed at 1000–1200 cm^{-1} wavelength correspond to C–H stretching vibration caused by carbohydrates such as cellulose and starch.

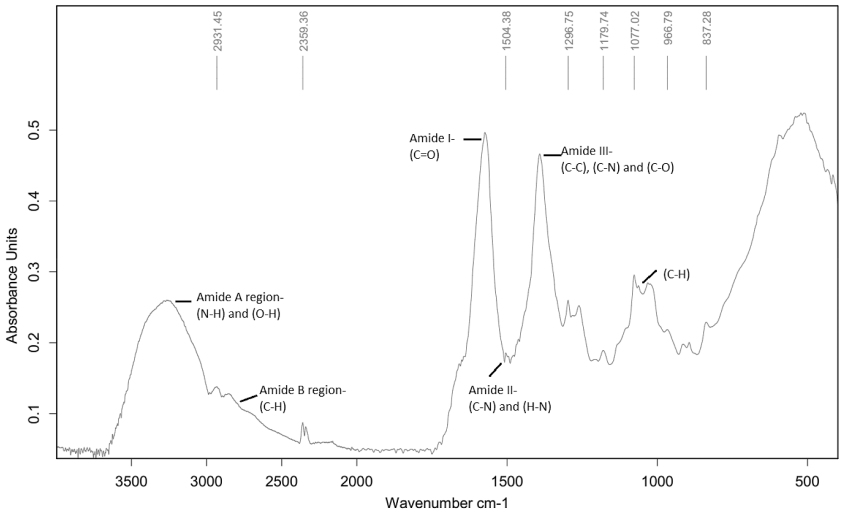

FIGURE 3 The FT-IR spectra of *Gracilaria dura* protein extract.

4 | CONCLUSION

The extraction conditions to obtain protein extracts from *G. dura* were successfully optimized by RSM and the optimum conditions were as follows; ultrasonic applying time of 257.57 s, enzyme/substrate ratio of 2.5, extraction time of 22.61 h. The protein extracts obtained from *G. dura* showed higher total phenolic contents and antioxidant activity than those of the other macroalgal species described in the literature. Additionally, the results of this study showed that *G. dura* protein extracts had more acceptable WAC, OAC, EA, and ES than those of other macroalgal or plant proteins. On the contrary, the foaming properties of protein extracts from *G. dura* were lower than those of other macroalgae. As a conclusion, protein extracts from *G. dura*, which is a low-cost and innovative source, may be employed as a functional food ingredient to boost antioxidant properties while also improving the techno-functional properties of food products. Therefore, in future studies, the effect of protein extracts from *G. dura* may be investigated on the techno-functional properties and antioxidant activity of foods enriched with algal protein extracts.

AUTHOR CONTRIBUTIONS

Ayşegül Bozdemir: Methodology; writing – original draft. **Eda Şensu:** Methodology; writing – original draft. **Emine Şükran Okudan:** Methodology. **Beraat Özçelik:** Methodology. **Aysun Yücetepe:** Methodology; supervision; writing – original draft; writing – review and editing.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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