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Studies on the Impact of Selected Pretreatments on Protein Solubility of *Arthrospira platensis* Microalga

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Abstract: *Arthrospira platensis* has emerged as a novel protein feed source since it contains high protein level and quality. However, this microalga presents a recalcitrant cell wall and its main proteins form protein-pigment complexes attached to the thylakoid membrane. The objective of the present study was to evaluate the influence of mechanical/physical pretreatments (bead milling, extrusion, freeze-drying, heating, microwave and sonication) on *A. platensis* protein solubility. Total protein content and solubility were assessed by Bradford method and SDS-PAGE quantification. Protein degradation was assessed through quantification of protein fractions (18–26 kDa, 40–48 kDa and others) in SDS-PAGE gels. Peptide formation was evaluated using the *o*-phthaldialdehyde assay. The results showed a decrease in total protein content in the supernatant with extrusion (0.07 to 1.42 mg/mL) and microwave pretreatments, and in the pellet with extrusion. Therefore, extrusion, followed by microwave, was the most effective pretreatment for *A. platensis* proteins denaturation and solubility. It is suggested that the extrusion process cause an irreversible denaturation and aggregation of the major microalga proteins (*c*-phycocyanin and allophycocyanin), with a strong decrease in their solubility. Therefore, extrusion could increase the bioaccessibility of *A. platensis* proteins and enable the incorporation of this microalga at higher levels in monogastric diets.

Keywords: *Arthrospira* sp.; microalgae; mechanical pretreatments; proteins solubility

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1. Introduction

Microalgae have increased their potential as novel nutrient sources because of their richness in proteins (values can vary from 8% in *Porphyridium cruentum* to 70% in *Arthrospira platensis* on a dry weight basis [1]), linolenic acid, fibers, vitamins, some minerals, pigments and several bioactive compounds [2].

Arthrospira platensis is a filamentous cyanobacterium that is known for its richness in proteins [3] and has been used as animal feed in aquaculture [4,5], for farm animals [6,7] and for pets [8,9]. Despite its great protein value, *A. platensis* has a recalcitrant cell wall that interferes with bioavailability and accessibility of nutrients and, therefore, the application of some pretreatments is necessary to increase protein availability [2]. As a cyanobacteria, *A. platensis* principal protein-pigment complex, phycocyanin, forms complexes with other phycobiliproteins, which constitutes the phycobilisomes. These complexes, which are linked to the thylakoid membrane, are composed by hexameric discs of *c*-phycocyanin attached to trimers of allophycocyanin, which are the core of phycobilisomes, together with hexameric discs of phycoerythrin. *c*-phycocyanin and allophycocyanin have, respectively, two or three chromophores per monomer and these monomers form higher aggregated structures that are linked dependently on solvent conditions [10,11]. This complex microalga structure influences bioaccessibility and digestibility of *A. platensis* nutrients [12], especially proteins with high biological value. So, it is important to find out alternative methods to overcome

this issue [3] and understand the effect of pretreatments on degradation of *A. platensis* cell wall.

Although there are various types of methods to disrupt the microalga cell wall in an attempt to improve nutrient accessibility (mechanical, physical, chemical and enzymatic), the mechanical and physical have been the most used for this purpose [13–15], with various reports describing the effect of some of them on the degradation of microalga proteins [1,16–19].

According to Krishnamoorthy, et al. [20], bead milling is commonly used for lipid extraction because of its effectiveness in alga cell disruption and easy operating procedures. This method damages the cells by applying forces that promote the collision between the beads and the cells, but the efficiency of this is closely related on the load and diameter of the beads [19,21]. Bead milling is usually used for improving the digestibility of microalgae in aquaculture [22]. Extrusion conditions, which include extreme temperature with high pressure, mixing and shear forces, can cause some damages in cell wall structure because it leads to physical/mechanical modification and disruption of biomass through passage in the extruder [23]. Wang, et al. [23] tested the extrusion method in *Nannochloropsis oceanica* with good results in cell disruption. Moreover, freeze-drying is recognized as an easy technique and is one of the most used pretreatments for lipid extraction because it helps solvent extraction [24]. One advantage of this pretreatment is the production of high valuable compounds without adverse effects on cellular constituents. Heating can be a good choice to increase the accessibility of algal proteins because of partial protein denaturation and the breakdown of proteins into peptides and amino acids that become more accessible for enzymes [25]. For microwave pretreatment, microalga cells are disrupted by an electromagnetic field, induced by microwave irradiation and heat generated by the combination of dielectric medium and polar molecules [19]. Sonication includes continuous compression and decompression of ultrasonic waves which provoke cavitation in the cell forming microbubbles that compress and collapse according to the intensity of the ultrasound [19]. These waves can cause cell wall disruption because of their high shear force [20].

The objective of this work was to assess and compare the effects of the most used physical and mechanical pretreatments (bead milling, extrusion, freeze-drying, heating, microwave and sonication) on *A. platensis* protein content and solubility.

2. Materials and Methods

2.1. Microalga Pretreatments

A. platensis dried powder obtained from Allmicroalgae Natural Products SA company (Pataias, Leiria, Portugal) was submitted to six different pretreatments following a specific protocol.

For bead milling, one bead was added *per* mL of microalga resuspension in 1 × phosphate buffered saline (PBS) buffer (BioWhittaker, Verviers, Belgium) at 20 mg/mL, and left under continuous rotation at 2000 rpm for 30 min in a shaker (Multi Reax Heidolph Instruments, Schwabach, Germany). Extrusion was done by Sparos company (Olhão, Algarve, Portugal) at 118 °C and 34 bars for 3 to 7 s with addition of water, 340 mL/min, and drying at 120 °C for 8–10 min. Then, microalga was resuspended with PBS buffer. The freeze-drying consisted in freezing *A. platensis* at −80 °C for 24 h and then freeze-drying it (Labogene, CoolSafe, Frilabo, Milheirós, Portugal) for another 24 h. In heating treatment, microalga stayed in dry heat (Melag, Geneststraße, Berlin, Germany) at 70 °C for 30 min. For microwave (Whirlpool, Household Microwave Oven, MI, USA) technique, alga suspension with PBS buffer was submitted to the keep warm mode until it boiled. Sonication (Bandelin ultrasonic homogenizer, Heinrichstraße, Berlin, Germany) conditions were 7 cycles at 70% power for 15 min with manual agitation of microalga suspension with PBS buffer at middle time. The chemical composition of non-treated *A. platensis* is presented in Table 1, which was conceded by Allmicroalgae company. The chemical composition was determined using routine and widespread methods [26]. Briefly, dry matter was analyzed

by drying a sample at 105 °C to a constant weight. The nitrogen content for crude protein determination was obtained using the Kjeldahl method. Ash content was determined after burning the sample at 525 °C. Crude fat value was obtained with Soxhlet extraction using petroleum. Gross energy and carbohydrates were determined by standardized calculations. Phycocyanin was extracted following the procedures described by Ritchie [27] and were analyzed by high-performance liquid chromatography.

Table 1. Chemical composition of *Arthrospira platensis*.

Nutritional Composition	
Energy (MJ/kg)	13.9
Crude protein (% dry matter)	62.6
Ash (% dry matter)	14.9
Crude carbohydrates (% dry matter)	6.06
Crude fibre (% dry matter)	9.78
Crude fat (% dry matter)	6.70
Pigment composition	
Phycocyanin (% dry matter)	11.2

2.2. Incubation for A. platensis after Pretreatments

A. platensis resuspension in PBS buffer at 20 mg/mL was incubated overnight with each pretreatment, in triplicate, in an orbital shaker (Sanyo MIR-220RU Refrigerated, Shaking Incubator) at 37 °C and 160 rpm, in a 24-well plate (VWR Chemicals, West Chester, PA, USA) (Figure 1) [28]. After incubation, the plate was centrifuged at 1500× *g*, 15 min, 4 °C, followed by the recovery of 1 mL supernatant and the resuspension with 1 mL PBS buffer to recover the pellet to 1.5 mL microtubes.

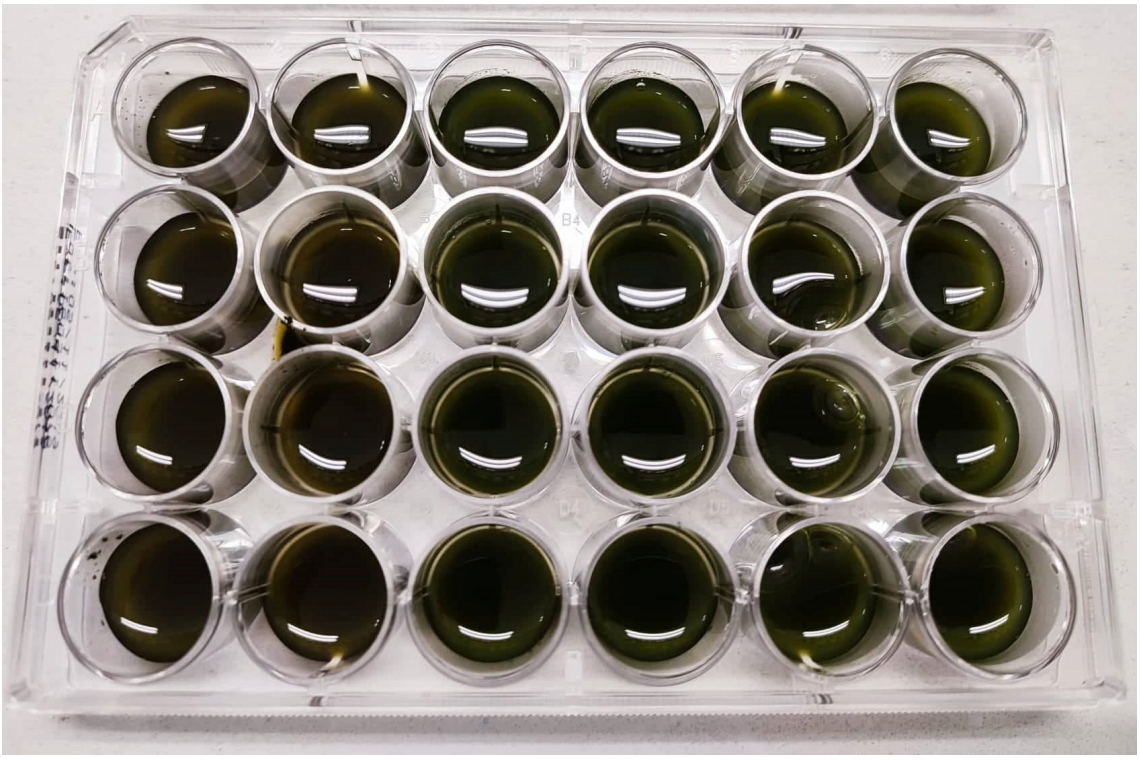

Figure 1. Typical aspect of the in vitro incubation of *Arthrospira platensis* with pretreatments in a 24-well plate.

2.3. Determination of Total Protein by Bradford Method

The Bradford method [29] was used to quantify solubilized total protein by spectrophotometry in the collected supernatant and pellet. Thus, 1.5 mL for Bradford solution (PanReac, AppliChem, ITW Reagents) was mixed with 30 µL of sample previously diluted in 1:3 factor with PBS buffer, and, after waiting 5 min at room temperature, the absorbance was read at 595 nm. A standard curve was done with bovine serum albumin at concentrations from 0.0125 to 1 mg/mL.

2.4. Electrophoretic Analysis of Proteins by SDS-PAGE

Microalga proteins, before and after pretreatments, were separated by 14% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). To evaluate protein fractions, 24 µL of each sample for supernatant or pellet (n = 5) was added to 6 µL of sample buffer, prepared according to Vizcaíno, et al. [30] but with some differences (prepared with 30% instead of 10% (v/v) β-mercaptoethanol). Then, the mixture was centrifuged, boiled for 5 min and added at 12 µL (9.00 µg of protein in each gel lane) into a 14% SDS-PAGE gel. The protein marker used was a low molecular weight marker (5 µL, about 1.95 µg of protein) with several protein fractions between 18.5 and 96 kDa (Nzytech, Lisbon, Portugal). Electrophoresis was done at a constant amperage of 12 mA per gel at 300 V (PowerPac Basic, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, the gels were colored with Coomassie brilliant blue and stayed overnight in distilled water before we processed the gel into an image using a software ChemiDoc XRS+ (Bio–Rad Laboratories, Inc., Hercules, CA, USA). The two most prominent protein fractions (18–26 kDa protein fraction 1, F1, and 40–48 kDa protein fraction 2, F2), other proteins and total protein, were quantified, in triplicate, by Image J software (NIH, Bethesda, MA, USA). The proportion of these fractions (F1, F2 and others) was also calculated relative to total protein.

2.5. Determination of Total Peptides by O-Phthaldialdehyde (OPA) Assay

The OPA spectrophotometric assay, used to measure free amino acids and peptides released from protein hydrolysis, was done according to Vizcaíno, et al. [30] and Sedighi, et al. [31], with some modifications. For each sample, 200 µL of each supernatant was mixed with 100 µL of 20% trichloroacetic acid for protein precipitation, followed by a centrifugation at 12,000× g for 15 min. Then, 200 µL of supernatant was added to 1 mL of OPA reagent, daily prepared according to Sedighi, et al. [31] and Church, et al. [32]. Blank was made with 200 µL distilled water, instead of supernatant sample. After incubation at room temperature for 5 min, the absorbance was read at 340 nm. A standard curve was done with peptone at ten concentrations between 5–500 µg/mL.

2.6. Statistical Analysis

Data were analysed using General Linear Models of SAS to perform analysis of variance (ANOVA) and the Tukey–Kramer method (PDIFF option) for multiple comparisons of adjusted least square means. The homogeneity of variances was tested using Levene's test. Values were considered significant when $p < 0.05$.

3. Results

Table 2 shows the effect of pretreatments on the degradation of *A. platensis* biomass proteins in the supernatant fraction.

Table 2. Effect of pretreatments on the solubility of *Arthrospira platensis* biomass proteins in the supernatant fraction (n = 5) (Values are presented as mean ± standard deviation).

Item	Pretreatments [1]							*p*-Value
	NoP	BM	ET	FD	HT	MW	SO	
Total protein (mg/mL)								
Bradford method	1.42 ± 0.112 ^{ab}	1.30 ± 0.061 ^{b}	0.07 ± 0.060 ^{c}	1.20 ± 0.105 ^{b}	1.29 ± 0.125 ^{b}	0.41 ± 0.054 ^{c}	1.72 ± 0.418 ^{a}	<0.001
SDS-PAGE gel	9.9 ± 1.58 ^{a}	10.1 ± 0.20 ^{a}	6.50 ± 1.035 ^{b}	9.6 ± 0.99 ^{a}	10.5 ± 1.61 ^{a}	7.0 ± 0.32 ^{b}	10.7 ± 0.54 ^{a}	<0.001
Proteins (mg/mL) in SDS-PAGE gel								
Proteins 18–26 kDa	2.41 ± 0.066 ^{ab}	2.35 ± 0.107 ^{ab}	1.44 ± 0.176 ^{c}	2.25 ± 0.030 ^{b}	2.74 ± 0.315 ^{a}	1.73 ± 0.256 ^{c}	2.65 ± 0.244 ^{a}	<0.001
Proteins 40–48 kDa	1.71 ± 0.094 ^{bc}	2.02 ± 0.205 ^{ab}	1.19 ± 0.188 ^{d}	1.94 ± 0.113 ^{ab}	2.10± 0.273 ^{a}	1.41 ± 0.057 ^{cd}	2.00 ± 0.056 ^{ab}	<0.001
Other proteins	5.74 ± 1.553 ^{a}	5.76 ± 0.152 ^{a}	3.87 ± 0.755 ^{bc}	5.39 ± 0.909 ^{abc}	5.61 ± 1.027 ^{ab}	3.81 ± 0.327 ^{c}	6.09 ± 0.588 ^{a}	0.001
Proteins (% total protein) in SDS-PAGE gel								
Proteins 18–26 kDa	22.3 ± 5.22 ^{ab}	23.2 ± 1.30 ^{ab}	18.7 ± 2.07 ^{b}	23.7 ± 2.49 ^{ab}	26.4 ± 1.17 ^{a}	24.9 ± 3.58 ^{a}	24.7 ± 2.72 ^{a}	0.011
Proteins 40–48 kDa	17.7 ± 3.15	19.9 ± 1.85	18.3 ± 0.95	20.3 ± 1.25	20.2 ± 0.56	20.3 ± 0.36	18.6 ± 0.52	0.047
Other proteins	60.0 ± 7.46 ^{ab}	56.8 ± 0.79 ^{ab}	63.0 ± 1.48 ^{a}	56.0 ± 3.64 ^{ab}	53.5 ± 1.70 ^{b}	54.9 ± 3.89 ^{b}	56.6 ± 3.13 ^{ab}	0.008
Proteins (PTRAT/PCON) in SDS-PAGE gel								
Total protein	nd	1.05 ± 0.196 ^{abc}	0.68 ± 0.207 ^{c}	1.00 ± 0.206 ^{abc}	1.07 ± 0.206 ^{ab}	0.72 ± 0.135 b ^{c}	1.12 ± 0.223 ^{a}	0.005
Proteins 18–26 kDa	nd	1.18 ± 0.110 ^{a}	0.69 ± 0.105 ^{b}	1.13 ± 0.046 ^{a}	1.23 ± 0.190 ^{a}	0.82 ± 0.038 ^{b}	1.17 ± 0.054 ^{a}	<0.001
Proteins 40–48 kDa	nd	0.98 ± 0.028 ^{ab}	0.60 ± 0.076 ^{c}	0.93 ± 0.021 ^{b}	1.13 ± 0.108 ^{a}	0.72 ± 0.102 ^{c}	1.10 ± 0.127 ^{ab}	<0.001
Other proteins	nd	1.08 ± 0.358	0.74 ± 0.320	1.01 ± 0.335	1.03 ± 0.336	0.71 ± 0.202	1.14 ± 0.426	0.237
Total peptides (µg/mL)								
o-phthaldialdehyde assay	42.7 ± 2.20 ^{a}	50.4 ± 9.20 ^{a}	24.6 ± 4.81 ^{b}	44.4 ± 9.84 ^{a}	38.5 ± 4.39 ^{a}	39.0 ± 5.02 ^{a}	43.4 ± 3.69 ^{a}	<0.001

[1] NoP: no pretreatment; BM, bead milling; ET, extrusion; FD, freeze-drying; HT, heating; MW, microwave; SO, sonication; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PTRAT, protein obtained with pretreatments; PCON, protein obtained with control; ^{a,b,c}, different letters in the same line indicate statistically significant differences; no letters within lines indicate no statistically significant differences; nd, not detected.

Total protein content (determined by the Bradford method) and total protein solubility (evaluated by SDS-PAGE gel quantification) significantly decreased ($p < 0.001$) with extrusion and microwave conditions. Concerning the protein quantification in the gel, only extrusion and microwave conditions caused a significant decrease ($p < 0.001$) in proteins with 18–26 kDa and other proteins. In addition, extrusion led to a significant ($p < 0.001$) decrease in proteins with 40–48 kDa, whereas an increase in this protein fraction was found with heating. However, no significant differences ($p > 0.050$) were found between pretreatments for the percentage of protein fractions. There was a significant decrease in the proportion of total protein ($p = 0.005$) and protein fractions with 18–26 kDa ($p < 0.001$) and 40–48 kDa ($p < 0.001$) relative to control (protein with pretreatments/protein with control; PTRAT/PCON) with extrusion and microwave pretreatments. Only extrusion led to significant decrease ($p < 0.001$) in peptide formation quantified by OPA assay.

Figure 2 presents representative images of SDS-PAGE gels ($n = 3$) for the effect of the pretreatments on algal proteins in the supernatant. It is possible to confirm the effect of extrusion on degradation of *A. platensis* proteins since the lanes concerning the influence of extrusion are clearly lighter than those with other pretreatments, indicating the disappearance of proteins.

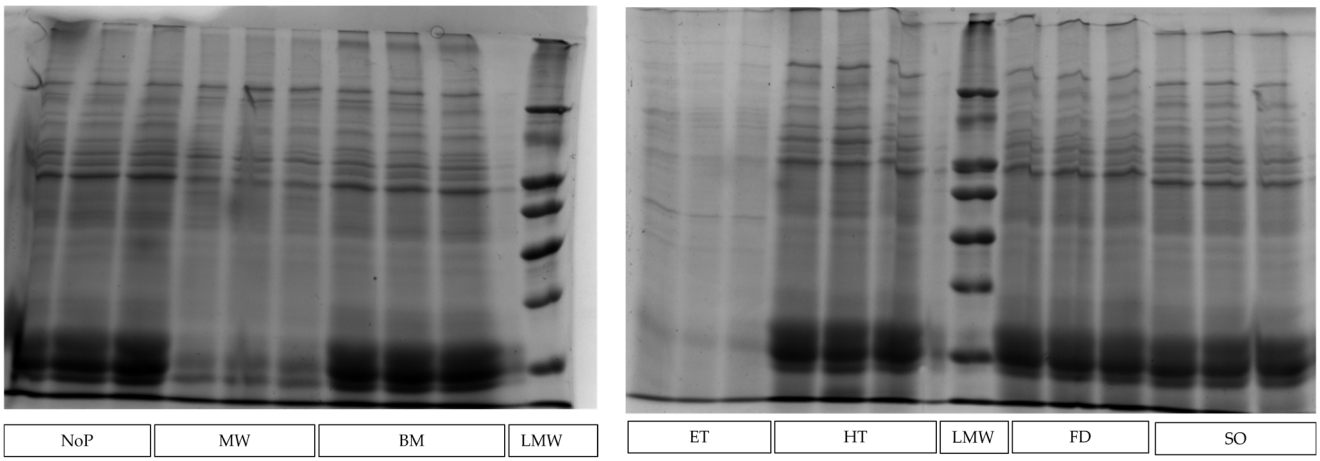

Figure 2. Images of SDS-PAGE gels of the supernatant fraction showing the effect of pretreatments on *Arthrospira platensis* protein amount and solubility after 24 h incubation ($n = 3$); LMW, low molecular eeight protein marker; BM, bead milling; ET, extrusion; HT, heating; FD, freeze-drying; MW, microwave; NoP, no pretreatment; SO, sonication.

The effect of pretreatments on *A. platensis* proteins in the pellet fraction is shown in Table 3, whereas the most representative SDS-PAGE gels ($n = 3$) are depicted in Figure 3. Only total protein content quantified by Bradford method decreased significantly ($p < 0.001$) with extrusion conditions compared to control. All the other variables were not significantly ($p > 0.050$) affected by treatments. Extrusion and sonication caused a decrease and increase, respectively, of the proportion of protein fraction with 18–26 kDa relative to the control assay.

Figure 3 shows no clear difference between the lanes of each pretreatment in the pellet fraction.

Table 3. Effect of pretreatments on the solubility of *Arthrospira platensis* biomass proteins in the pellet fraction (n = 5) (Values are presented as mean ± SD).

Item	Pretreatments [1]							*p*-Value
	NoP	BM	ET	FD	HT	MW	SO	
Total protein (mg/mL)								
Bradford method	0.83 ± 0.094 ^{bc}	1.13 ± 0.312 ^{b}	0.03 ± 0.039 ^{d}	0.71 ± 0.091 ^{c}	0.67 ± 0.101 ^{c}	1.13 ± 0.076 ^{b}	2.36 ± 0.246 ^{a}	<0.001
SDS-PAGE gel	11.5 ± 0.59	12.9 ± 0.79	13.8 ± 5.07	11.5 ± 1.85	11.3 ± 0.31	13.6 ± 0.64	10.2 ± 1.39	0.115
Protein quantification (mg/mL) by SDS-PAGE gel								
Proteins 18–26 kDa	2.23 ± 0.026	2.40 ± 0.057	2.14 ± 0.237	2.29 ± 0.132	2.16 ± 0.067	2.38 ± 0.080	2.40 ± 0.337	0.075
Proteins 40–48 kDa	1.95 ± 0.032	2.14 ± 0.088	1.97 ± 0.255	1.98 ± 0.264	1.96 ± 0.045	2.20 ± 0.090	2.19 ±0.440	0.279
Other proteins	7.36 ± 0.591	8.33 ± 0.673	9.66 ± 5.041	7.20 ± 1.456	7.16 ± 0.214	8.99 ± 0.698	5.60 ± 2.054	0.110
Protein proportion (% total) by SDS-PAGE gel								
Proteins 18–26 kDa	19.3 ± 0.87 ^{ab}	18.7 ± 0.80 ^{ab}	16.7 ± 4.01 ^{b}	20.3 ± 2.51 ^{ab}	19.1 ± 0.15 ^{ab}	17.6 ± 1.14 ^{b}	24.2 ± 6.39 ^{a}	0.020
Proteins 40–48 kDa	16.9 ± 0.63	16.6 ± 0.29	15.9 ± 0.59	17.4 ± 1.20	17.4 ± 5.55	16.3 ± 1.06	22.2 ± 7.31	0.129
Other proteins	63.7 ± 1.91	64.7 ± 1.37	67.3 ± 9.53	62.4 ± 3.09	63.5 ± 0.22	66.2 ± 2.30	53.6 ± 13.69	0.056
Protein proportion (PTRAT/PCON) by SDS-PAGE gel								
Total protein	nd	1.11 ± 0.037	1.21 ± 0.505	0.99 ± 0.126	0.98 ± 0.071	1.18 ± 0.083	0.88 ± 0.093	0.169
Proteins 18–26 kDa	nd	1.08 ± 0.014	0.96 ± 0.117	1.03 ± 0.049	0.97 ± 0.040	1.07 ± 0.037	1.08 ± 0.162	0.132
Proteins 40–48 kDa	nd	1.10 ± 0.051	1.01 ± 0.140	1.02 ± 0.144	1.00 ± 0.021	1.13 ± 0.052	1.12 ± 0.214	0.385
Other proteins	nd	1.13 ± 0.044	1.36 ± 0.812	0.97 ± 0.150	0.98 ± 0.102	1.23 ± 0.134	0.75 ± 0.236	0.145

[1] NoP: no pretreatment; BM, bead milling; ET, extrusion; FD, freeze-drying; HT, heating; MW, microwave; SO, sonication. SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PTRAT, protein obtained with pretreatments; PCON, protein obtained with control; ^{a,b,c}, different letters in the same line represent means with statistically significant differences; no letters within lines indicate no statistically significant differences; nd, not detected.

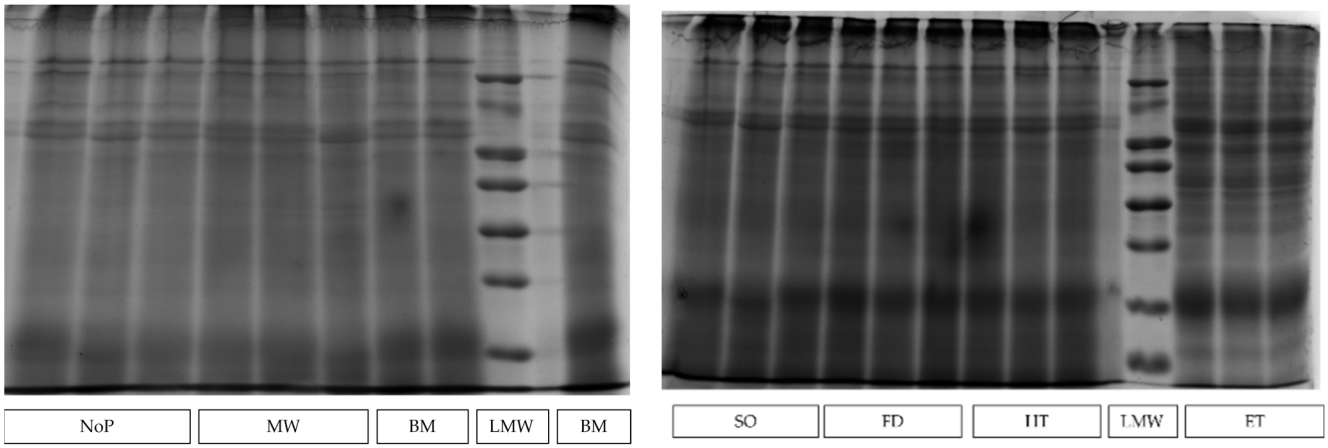

Figure 3. Images of SDS-PAGE gels of the pellet fraction showing the effect of pretreatments on *Arthrospira platensis* protein amount and solubility after 24 h incubation (n = 3); LMW, low molecular weight protein marker; BM, bead milling; ET, extrusion; HT, heating; FD, freeze-drying; MW, microwave; NoP, no pretreatment; SO, sonication.

4. Discussion

The present results showed that most of the mechanical/physical pretreatments studied here (bead milling, freeze-drying, heating and sonication) did not affect protein amount and solubility. However, both extrusion and microwave pretreatments were effective on the reduction of solubility of *A. platensis* proteins, with significant decreases of total protein and proteins fractions with 18–26 kDa and 40–48 kDa. Nevertheless, the lower content of proteins (assessed by the Bradford method) in the pellet fraction of extruded microalga relative to the microwave-treated indicate a stronger impact of extrusion on protein denaturation and aggregation.

Extrusion conditions seem to drastically change *A. platensis* protein's structure and solubility when these were submitted to the severe conditions of temperature and pressure of this mechanical/physical pretreatment. Visualizing the gels (Figures 2 and 3), it is possible to see that the lanes referred to extrusion conditions in the supernatant fraction disappeared and are, instead, present in the pellet fraction, which seems to indicate a strong protein aggregation after applying this pretreatment, although there were no significant differences in the total protein quantified in SDS-PAGE gel (Table 3). In fact, Böcker, et al. [10] reported that *c*-phycocyanin and allophycocyanin, which correspond to protein fraction with 18–26 kDa, are sensitive to thermal processing, suffering modification in their protein structure and properties. Indeed, high temperatures can lead to the denaturation of proteins and the loss of secondary and tertiary protein structures. Such high temperatures were achieved, in the present study, with extrusion and microwave pretreatments, which can explain the reduction of total protein in the algal supernatant with both pretreatments.

The aggregation of algal proteins, an irreversible structure, with extrusion conditions, and to a lesser extent by microwave conditions, seems to promote their accessibility to peptidases, thus improving digestibility [33]. Carbonaro, et al. [33] attested that the improved digestibility of chickpea proteins, after their heat-induced denaturation, was due to the enhanced accessibility of susceptible sites to proteolysis of denaturized/aggregated proteins. Moreover, according to Wang, et al. [23], extrusion is a good method for improving the extraction of bioactive compounds from *Nannochloropsis oceanica*, increasing 74.3% of polyunsaturated fatty acids and 20.5% of essential amino acids in comparison with control. The authors observed a minimum decrease in protein yield with extrusion conditions, probably influenced by shear and heat conditions, which affected protein structure or even caused protein degradation. It seems that lower water content in extrusion conditions is beneficial for microalga cell disruption and consequently increasing soluble protein formation. Moreover, extrusion conditions tend to increase algal essential amino acids with improvement of protein solubility due to the degradation of proteins into amino acids.

However, it is important to consider the possible occurrence of Maillard reaction, involving free protein amino groups and reducing the sugar carbonyl groups, which reduces amino acid availability [23]. Moreover, *Arthrospira maxima* extruded pellets led to high in vivo protein digestibility (up to 82.5%) when fed to fish [22].

Microwave pretreatment also had a significant effect on protein amount and solubility in previous studies. This pretreatment has been tested in some microalgae (*Chlorella* sp. and *Scenedesmus*), but specifically for lipid extraction without any focus on proteins [19,34–36]. Piasecka, et al. [35] analyzed the fatty acid profile of *Chlorella protothecoides* and concluded that microwave pretreatment increased the content of palmitic acid and modified the unsaturated/saturated fatty acids ratio. Viner, et al. [36] tested microwave radiation with distilled water in *Scenedesmus* sp., finding very good results with respect to the release of neutral lipids and free fatty acids. Only one study, so far, reported the effect of a microwave-assisted technique in protein extraction, showing that microwave pretreatment was a good method for disruption of *Chlorella vulgaris* cell wall with significant results on proteins recovery (increase in 2.54 times in comparison with three phase partitioning extraction) [37].

Although bead milling, in theory, is a good method for cell disruption, and possible to apply in a large-scale [21], it was not significantly efficient in the present study, which can be attributed to the characteristics of the beads. Freeze-drying conditions also did not cause any differences on total protein amount or solubility, although they have been tested for improvement of soluble proteins extraction with good results for *C. vulgaris* [38]. A study concerning the effect of bead milling and freeze-drying applied to microalga *Nannochloropsis gaditana* on apparent digestibility coefficients in fish showed increased digestibility values with bead milling but no differences with freeze-drying (75.6% and 59.8%, respectively, in comparison with control, 59.3%) [12].

Concerning heating pretreatment, Abbassi, et al. [39] tested five different temperatures (20, 40, 60, 80 and 100 °C) with two pressure levels (10 and 50 bar) and the results confirmed that the temperature–pressure combination has an effect on cell wall disruption. These results did not occur in our work, probably because of the low heating temperature (70 °C) that was not sufficient for protein denaturation and to decrease in its solubility [10,11]. Although, in the present study, no significant differences were found with sonication, possibly due to the conditions used (combination of time, power and cycles), this method has been successfully applied to disrupt cells of various microalga species; however, it was not effective when microalgae were in very high concentrations [21]. Martínez-Sanz, et al. [40] tested the influence of ultrasound in the extraction of proteins, carbohydrates and lipids of three different species of microalga, such as *A. platensis,* with production of 27% of proteins. Moreover, the authors concluded that sonication conditions promoted the release of intracellular compounds without damaging the cell wall integrity.

5. Conclusions and Future Perspectives

Data presented here indicate that the extrusion process, followed by the microwave methodology, was the pretreatment that mostly affected *A. platensis* proteins denaturation and solubility, especially for the main protein fractions (18–26 kDa and 40–48 kDa). Extrusion conditions seem to cause irreversible microalga protein denaturation and aggregation, with a strong decrease in their solubility, especially for *c*-phycocyanin and allophycocyanin. Therefore, it could be the most efficacious mechanical/physical pretreatment for improving *A. platensis* cell disruption and the bioaccessibility of proteins.

The other mechanical/physical pretreatments studied here (bead milling, freeze-drying, heating and sonication) did not affect *A. platensis* protein denaturation and solubility under these experimental conditions.

Further studies could elucidate the impact of other extrusion conditions, alone or in combination with other mechanical/physical methods, or even enzymatic pretreatments, on protein solubility and degradation. Moreover, considering the applicability of extrusion at an industrial scale, the effect of extrusion on the protein bioaccessibility of *A. platensis* in monogastric animals needs to be established. Therefore, in vivo trials with monogastric

animals fed with high incorporation levels (up to 15–20% feed) of this extruded microalga are being performed by our team to test the effect of this pretreatment on *A. platensis* digestibility.

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