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Development of Enzyme-Based Cosmeceuticals: Studies on the Proteolytic Activity of *Arthrospira platensis* and Its Efficient Incorporation in a Hydrogel Formulation

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Abstract: Microalgae are a valuable source of enzymes and active ingredients due to their biochemical variability, health-beneficial properties, lower production cost and viability on an industrial scale. In addition, the growing demand for sustainable products constitutes microalgae as a viable resource for the development of a range of innovative products. In the present work, the proteolytic activity of *Arthrospira platensis* lysate was assessed and characterized using kinetics analysis and zymography employing different substrates, pH values and divalent ions. The results suggest the presence of two main proteolytic enzymes in the lysate. In addition, a rapid and easy purification protocol of the proteolytic activity, under mild conditions, was developed using a polyethyleneglycol (PEG)/phosphate aqueous two-phase system (ATPS). The proteolytic activity of *A. platensis* lysate was used to develop a hydrogel formulation as an enzyme-based cosmeceutical, with potential application as a topical exfoliating agent. The incorporation of the *A. platensis* extract in the developed hydrogel formulation significantly improved its operational stability over time, which is a significant advantage in enzyme-based product development.

Keywords: cosmeceuticals; protease; *Arthrospira platensis*; aqueous two-phase system; hydrogel

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1. Introduction

Proteases are involved in the regulation of numerous physiological pathways, they show selectivity for a wide range of substrates and entail a variety of structural features [1–3]. They are utilized in a variety of industrial processes and correspond to 60% of all industrial enzymes [4–8].

The vast biodiversity of marine ecosystems is considered a natural reservoir for the identification of useful biocatalysts with industrial and pharmaceutical applications [9–12]. These enzymes are often characterized by properties related to their environment, such as tolerance to salinity, cold adaptivity, barophilicity and increased thermal stability [10,13–15]. Therefore, marine microorganisms are a potential source of industrial enzymes due to ease in genetic manipulation, ability for mass culture, biochemical variability, catalytic properties, substrate selectivity, lower cost and viability at an industrial scale [12,16]. The high activity and stability of endogenous proteases from marine microorganisms, have established them as a promising ecological asset in medicine, chemical industry and food processing [17–19]. In addition, there is a growing demand for environmentally friendly and sustainable products; thus, marine organisms constitute a viable and promising bio-based resource [20–22].

The filamentous cyanobacterium *Arthrospira platensis*, also known as spirulina, is of particular interest in biotechnology since it is rich in bioactive compounds as well as natural dyes, and their mass mainly consists of proteins at 55 to 70% [23,24]. *Arthrospira* dried biomass has been authorized as "GRAS" (generally recognized as safe) by the US Food and Drug Administration (FDA) and is widely used in product development [25]. *A. platensis*

is commercially available as a dietary supplement as it has been shown to affect many biological processes since it displays a range of bioactivities such as anti-allergic, anti-inflammatory, antioxidant, antifungal, antibacterial and antiviral properties [26–29]. It has been used for the improvement of health-beneficial properties in pharmaceuticals [30–33] and cosmetics [22]. Furthermore, it entails a vast potential for developing sustainable applications, for example, in biofuel production and wastewater treatments for the removal of heavy metals and toxic compounds from water [34–40]. Regarding the proteolytic content of *A. platensis*, there has been detected a serine protease associated with selective phycobiliprotein proteolysis [41], an arginine protease [42] and an extracellular neutral protease (possibly cysteine protease) [43].

Algae-derived secondary metabolites are known for their skin benefits [44]. The cosmetics industry extensively uses compounds or extracts from natural sources due to the global trend toward environmentally sustainable bio-based products [22]. Algae have developed protective systems against free radicals and oxidative stress, resulting in the production of useful compounds for the cosmetic industry, for example, against ultraviolet radiation [22,45]. *Arthrospira* has been recently employed in cosmetics due to its moisturizing, brightening, antibacterial and antioxidant properties, as well as in wound healing applications [22]. In dermatology, the administration of proteolytic enzymes, such as papain, collagenase and bromelain, is investigated for wound healing and removal of necrotic tissues from wounds and burns [46–51]. Debridement is the most common application of enzymatic wound treatments, where collagenases and some serine and cysteine proteases are commonly used [52].

The incorporation of cosmetic-oriented proteolytic enzymes is proposed for applications in skin cleansing, hydration and restoration of the skin barrier in conjunction with the improvement of wound inflammation [53–56]. The use of proteases in skin care products is not intended to disable the pro-inflammatory endogenous proteins but to soften their effects when needed; thus, proteolytic enzymes entail a protective role in the direct or indirect modification of the host's tissues [56,57]. The type and concentration of proteases should be appropriate to attenuate the effects of endogenous proteins produced before the induction of inflammatory phenomena and have negative consequences when they are overactive [56,58]. Therefore, the use of proteases can potentially improve the hydration and repair of the skin barrier with a more therapeutic approach, compared to conventional skin care products [59]. In cosmetics, proteases are mainly used to promote exfoliation and ultimately increase absorption of water and other cosmetic ingredients [60,61]. Proteases can improve the skin's appearance through exfoliation; however, the widely used bromelain, papain and chymotrypsin have been linked to allergic reactions [62]. Commercial proteases for cosmetic use can be obtained by recombinant DNA technology or from alternative sources. Indicatively, the serine protease subtilisin produced by *Bacillus licheniformis* is an effective skin exfoliator [63]. This microorganism has also been used for the commercial production of keratinase [64,65], which may be used on stretch marks and scars and help regenerate epithelial cells by accelerating healing.

In the present work, the proteolytic activity of *Arthrospira platensis* lysate was assessed and characterized under different conditions, as well as fractionated using an ATPS. The algal lysate was eventually used to develop a stable hydrogel formulation with potential dermal use, where its proteolytic action may contribute to the local improvement of the epidermal surface. This venture originated from the fact that marine resources are considered safe and with negligible cytotoxicity to humans, while they are rich in bioactive substances with skin benefits and especially in the treatment of skin rashes and signs of aging [22,66,67].

2. Materials and Methods

2.1. Materials

Arthrospira platensis lyophilized cells were obtained from a local dietary supplement (Cretan Spirulina, Greece or Ecoduma, Austria). Coomassie brilliant blue G-250 that was

used in zymography was purchased from Merck (Darmstadt, Germany). The analytical grade salts and compounds that were used were obtained from Sigma-Aldrich (Burlington, MA, USA). Dermosoft® OMP was supplied by Dr. Straetmans GmbH (Hamburg, Germany) and Lecigel™ by IFF Lucas Meyer Cosmetics (Quebec, QC, Canada).

2.2. Methods

2.2.1. Extraction from Lyophilized *Arthrospira platensis*

Lyophilized *A. platensis* cells were initially in flake form. Extraction was performed in a mortar at 4 °C for at least 5 min. Extraction of 1 g of flakes was carried out in 4 mL of appropriate buffer (10 mM sodium acetate, 5 mM calcium acetate, pH 7.5). This procedure was followed by ultra-sonication in a water bath for 10 min. The cell lysate was obtained after centrifugation at 8000 rpm for 15 min.

2.2.2. Synthesis of Azoprotein Substrates

Synthesis of azoprotein substrates was accomplished by dissolving 20 g of protein (gelatin, casein or collagen) in 275 mL H_2O, containing 4 g $NaHCO_3$. Following solubilization, the temperature was raised to 54 °C. Then, 0.01 mol of sulfanilic acid was dissolved in 30 mL of H_2O which contained 0.01 mol NaOH and 0.01 mol $NaNO_2$. After the addition of 0.02 mol HCl, the solution was stirred to complete the solubilization of all compounds. Afterward, 0.02 mol NaOH was added, and the solution was mixed vigorously with the protein solution (gelatin, casein or collagen). The resulting mixture had a red color and was dialyzed against four liters of 0.01% *w/v* sodium azide (NaN_3), which was used as a preservative. After dialysis, the azoprotein solution was lyophilized and stored at −20 °C.

2.2.3. Determination of Proteolytic Activity with Azoprotein Substrate

Proteolytic activity was determined with azocasein, azogelatin and azocollagen as substrates. The determination of proteolytic activity was based on a previously published method as reported by Coêlho et al. [68]. During enzymatic hydrolysis of the azoprotein substrate (0.2% *w/v*), the dye was released into the solution while the proteins precipitated in the presence of (10% *v/v*) trichloroacetic acid (TCA). The color change was determined by the absorbance at 440 nm. One unit of enzyme activity was defined as the amount of enzyme that causes a 0.01 increase in absorbance at 440 nm. The reaction buffer for proteolytic activity determination was 0.1 M Tris-HCl, pH 8.5, unless stated otherwise.

2.2.4. The Effect of pH and Metal Ions on Proteolytic Activity

The effect of pH on the proteolytic activity of the *A. platensis* lysate was determined in the range 6.0–9.0 (0.1 M Tris-HCl) using azocasein, azogelatin or azocollagen (2% *w/v*) as substrates. The effect of different metal ions ($CuSO_4$, $CoCl_2$, $C_4H_6FeO_4$, $MnCl_2$, $MgSO_4$, $ZnCl_2$, $CaCl_2$) on the proteolytic activity of the *A. platensis* lysate was determined using azocasein or azogelatin as substrate (2% *w/v*) in 0.1 M Tris-HCl buffer pH 6.5 or pH 9.0. The effect of pH on the proteolytic activity of the ATPS bottom phase was determined in the range of 3.6 to 11.5 using as substrate, azocasein. The buffers used were: 0.1 M acetate solution for pH 3.6 to 6.0; 0.1 M Tris-HCl for pH 6.5 to 9.0; 0.1 M Glycine-NaOH for pH 9.5 to 11.5.

2.2.5. Zymography

Zymography was carried out using a modified SDS-PAGE procedure under non-reducing conditions, therefore without using reducing agents (e.g., β-mercaptoethanol) or heat treatment of the samples. The 9% (*w/v*) acrylamide separating gel contained 0.1% (*w/v*) copolymerized casein in the matrix in order to allow the detection of proteolytic activity after staining. After the standard electrophoretic run, the SDS was removed by incubating the gel in 2.5% (*v/v*) Triton X-100 for 40 min at room temperature. Afterward, the gel was incubated in a suitable buffer (50 mM Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, pH 6.5; or 50 mM Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, pH 8.5) at 37 °C for 16–20 h under gentle

shaking. The next day, the gel was immersed in a 0.05% (*w/v*) Coomassie brilliant blue G-250 staining solution (25% (*v/v*) methanol, 10% (*v/v*) acetic acid) for 1 h. Then, it was destained in 4% (*v/v*) methanol and 8% (*v/v*) acetic acid solution for 2 h under gentle stirring. The proteolytic activity appeared as clear bands on a blue background.

2.2.6. Aqueous Two-Phase System (ATPS)

Separation of the proteolytic activity of *A. platensis* lysate was achieved using an aqueous two-phase partition system (ATPS) composed by polyethylene glycol (PEG)/phosphate buffer. The optimal molecular weight of PEG, the pH and the salt concentration in the system [69] were evaluated in order to optimize the separation and purification efficiency of the method. Initially, the effect of PEG's molecular weight on separation was determined with the following conditions: (a) 27.4% (*w/w*) PEG 1500 (stock solution 80% (*w/w*)), 32.7% (*w/w*) phosphate pH 7.0, 30% (*w/w*) ddH_20, 10% (*w/w*) sample, and (b) 33.8% (*w/w*) PEG 3000 (stock solution 50% (*w/w*)), 36.2% (*w/w*) phosphate buffer pH 7.0, 20% (*w/w*) ddH_2O, 10% (*w/w*) extract. The ATPS solutions were gently stirred in the dark for one hour, and then they were centrifuged at 2500 *g* for 10 min for phase separation. The tested conditions were evaluated based on the proteolytic activity and purity of the top and bottom phases of the system. Moreover, the effect of pH of the phosphate buffer (11.2% (*w/w*) potassium monobasic phosphate, 28.8% (*w/w*) potassium dibasic phosphate) in range 6.5–8.5 was studied, as well as the impact of the inclusion of a neutral salt (3.5% (*w/v*), 7% (*w/v*) and 10% (*w/v*) NaCl) in the phosphate buffer.

The distribution of biomolecules in the ATPS phases depends on their equilibrium. In this work, the distribution factor K was calculated based on the estimated proteolytic activity (A) in each phase of the system (A_{top}, A_{bottom}) [70,71].

$$K = A_{top}/A_{bottom}$$

The purification efficiency (%) and purity (PF) were calculated according to the following equations [72–75].

$$PF_{top} = SA_{top}/SA_{crude}, PF_{bottom} = SA_{bottom}/SA_{crude}$$

where SA_{top} and SA_{bottom} correspond to the specific activity of the top and bottom phases of ATPS, and SA_{crude} is the specific activity of the lysate before ATPS (crude extract).

$$Efficiency_{top}\ (\%) = (V_{top}\ C_{top})/[(V_{top}\ C_{top}) + (V_{bottom}\ C_{bottom})]$$

$$Efficiency_{bottom}\ (\%) = (V_{bottom}\ C_{bottom})/[(V_{top}\ C_{top}) + (V_{bottom}\ C_{bottom})]$$

where C_{top} and C_{bottom} correspond to the concentration of proteins distributed in the top and bottom phases, respectively. V_{top} and V_{bottom} are the volume of the top and bottom phases.

The distribution coefficient of the proteins (K_p) is defined as the ratio of the protein concentration in the top phase (C_{ptop}) to the corresponding concentration in the bottom phase ($C_{pbottom}$). Protein concentration was determined using the Bradford method with albumin as a standard protein [76]. The ratio of the distribution coefficients (K/K_p) evaluates the selectivity of the system (S: Selectivity).

$$Selectivity\ (S) = K/K_p = (A_{top}/A_{bottom})/(C_{ptop}/C_{pbottom})$$

2.2.7. Preparation of a Hydrogel with Proteolytic Activity with *A. platensis* Lysate as the Main Ingredient

Due to the growing research interest in proteases with application in clinical dermatology and cosmetology [45,56], a hydrogel with proteolytic activity was developed by utilizing the *A. platensis* lysate as the main ingredient. The composition of the gel was based on the study of Matro et al. [77], so that most of the hydrogel formulation corresponds to the *A. platensis* lysate. The hydrogel was prepared by the addition of 3.4% (*w/v*) of the mois-

turizing and antimicrobial agent Dermosoft® OMP (phenylpropanediol, 1,2-octanediol, phenylpropanol) in the *A. platensis* lysate. Then, 3.8% (*w/v*) Lecigel™ (sodium acrylates copolymer and lecithin) was added, and the mixture was homogenized, thus resulting in the hydrogel formation. The commercial ingredients that were used in the preparation are suitable for dermal use. The formulated hydrogels were stored in a container protected from light.

The entrapped proteolytic activity in the hydrogel was assayed using azocasein (2% *w/v*) as substrate. The hydrogel was dissolved prior to the assay, and therefore a sufficient amount (mg) was weighed and added to corresponding volume of the reaction buffer (0.1 M Tris-HCl, pH 8.5) at 1:1 ratio.

2.2.8. Enzyme Stability

The stability of the proteolytic activity was determined after storage at 4 °C, 25 °C and 40 °C. The stability of the proteolytic activity was studied using: (a) *A. platensis* lysate in 10 mM sodium acetate, 5 mM calcium acetate, pH 7.5; (b) the bottom phase of ATPS; (c) the hydrogel that was prepared using the *A. platensis* lysate. The proteolytic activity was determined using azocasein(2% *w/v*) as a substrate.

3. Results and Discussion

3.1. Effects of Different pH, Metal Ions and Different Protein Substrates on the Proteolytic Activity of A. platensis Lysate

The effect of different pH values on the proteolytic activity of the *Arthrospira platensis* lysate was determined in the range of 6 to 9. The assays were performed using azocasein, azogelatin and azocollagen as substrates. The highest specific activity was established using azocasein as substrate, followed by azogelatin and azocollagen (Figure 1). Noteworthy, the proteolytic activity toward azocollagen was the lowest, yet not negligible, suggesting the potential cosmeceutical use of this enzyme system. Similar proteolytic activity, using azocollagen as substrate, is also displayed by the popular plant proteases bromelain [78] and papain [79]. Higher specific activity was observed at pH 9 for all three substrates used in the assay. Interestingly, a second clear peak in specific activity was also observed at pH 6.5 using azocasein as a substrate. This is possibly related to the presence of two proteolytic enzymes in the lysate that exhibit different pH optimums. Therefore, zymography with casein as a substrate was carried out using incubation buffers with pH 6.5 and 8.5 (Figure 2). Zymography at pH 6.5 allowed the detection of two protein zones with molecular masses at approximately 75 kDa and 35 kDa, whereas only the protein zone of 75 kDa was detected in the zymograph that was incubated at pH 8.5. Indicatively, an 80 kDa arginine protease has been identified in *A. platensis*, which catalyzes the hydrolysis of phycocyanin [41,42]. Moreover, a 72 kDa serine metalloprotease with fibrinolytic activity, which was favored by the presence of Fe^{2+}, and with optimal pH 6.0 has been previously studied [80].

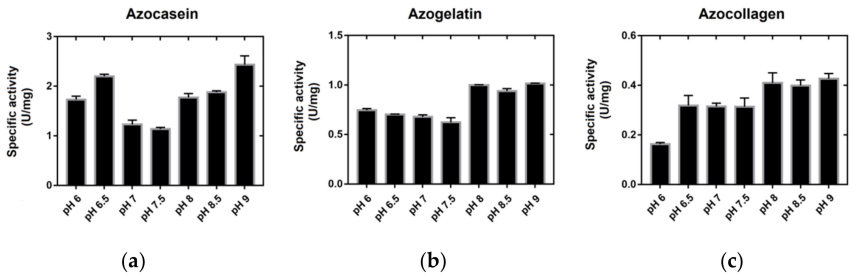
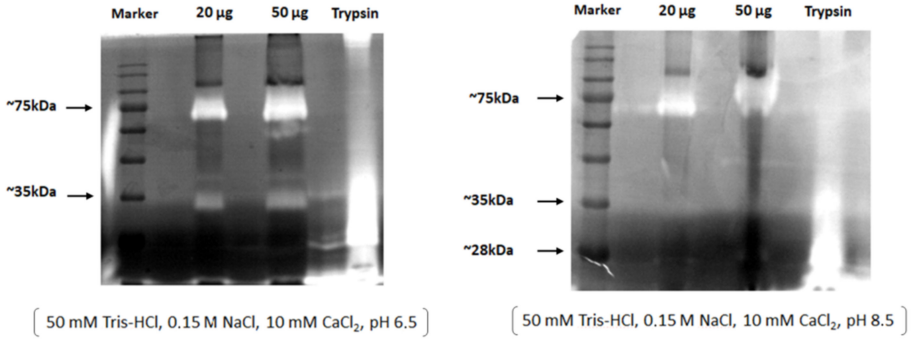


Figure 1. Specific activity (U/mg) of *A. platensis* lysate in pH 6 to 9 and substrate (**a**) azocasein, (**b**) azogelatin and (**c**) azocollagen. The data represent the mean ± SD (N = 3).



(**a**) (**b**)

Figure 2. Zymographs of *A. platensis* lysate (20 µg and 50 µg) with casein as a substrate, after 16 h of incubation in 50 mM Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$ with (**a**) pH 6.5 and (**b**) pH 8.5. Trypsin was used as a control (0.5 Units).

The effects of metal ions (Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}) on the proteolytic activity of *A. platensis* lysate were evaluated (2 and 5 mM final concentration) at pH 6.5 and 9.0, using azocasein or azogelatin (Figure 3). Significant loss of enzyme activity was observed in the presence of Cu^{2+} and Zn^{2+} with azocasein as substrate (Table S1). On the other hand, an increase in proteolytic activity was detected in the presence of Mg^{2+} and Ca^{2+}, mainly at 5 mM final concentration. In the case of azogelatin, the results indicate only a slight improvement with the addition of Mg^{2+}, while no significant effect was observed in the presence of Ca^{2+} in the reaction. The metal ions Mn^{2+} and Co^{2+} did not significantly affect the enzymatic activity with either substrate. Cu^{2+} showed high inhibition effect in all conditions.

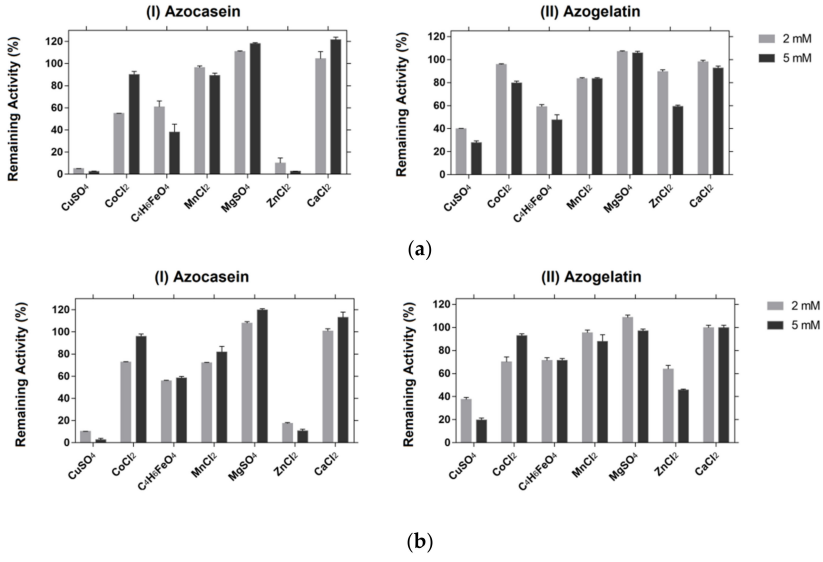


Figure 3. Effect of metal ions on the proteolytic activity. Remaining activity (%) of *A. platensis* lysate after determination of enzyme activity with (I) azocasein and (II) azogelatin as substrates at (**a**) pH 6.5 and (**b**) pH 9.0, with 2 mM and 5 mM final concentration of metal ions. Proteolytic activity in absence of metals is defined as 100%. The data represent the mean ± SD (N = 3).

Zymography with casein as a substrate indicated that the protein band at 35 kDa is more faintly destained at both pH 6.5 and 8.5, in the absence of Ca^{2+} in the incubation buffer, than in the zymograph at pH 6.5 which contained Ca^{2+} (Figures 2 and 4). Therefore, the proteolytic activity of the 35 kDa protein is probably activated in the presence of Ca^{2+}. Furthermore, the 35 kDa protein zone was faintly destained in the zymographs after incubation at pH 8.5 with either $CaCl_2$ or $MgSO_4$, compared to the zymograph at pH 6.5 with Ca^{2+} (Figure 4). However, since the presence of Ca^{2+} and Mg^{2+} led to the improvement in the specific activity in the activity assay (Figure 3), it is conceivable to

propose that the 35 kDa enzyme corresponds to a protease that is activated by Ca^{2+} and possibly Mg^{2+} at optimum pH 6.5.

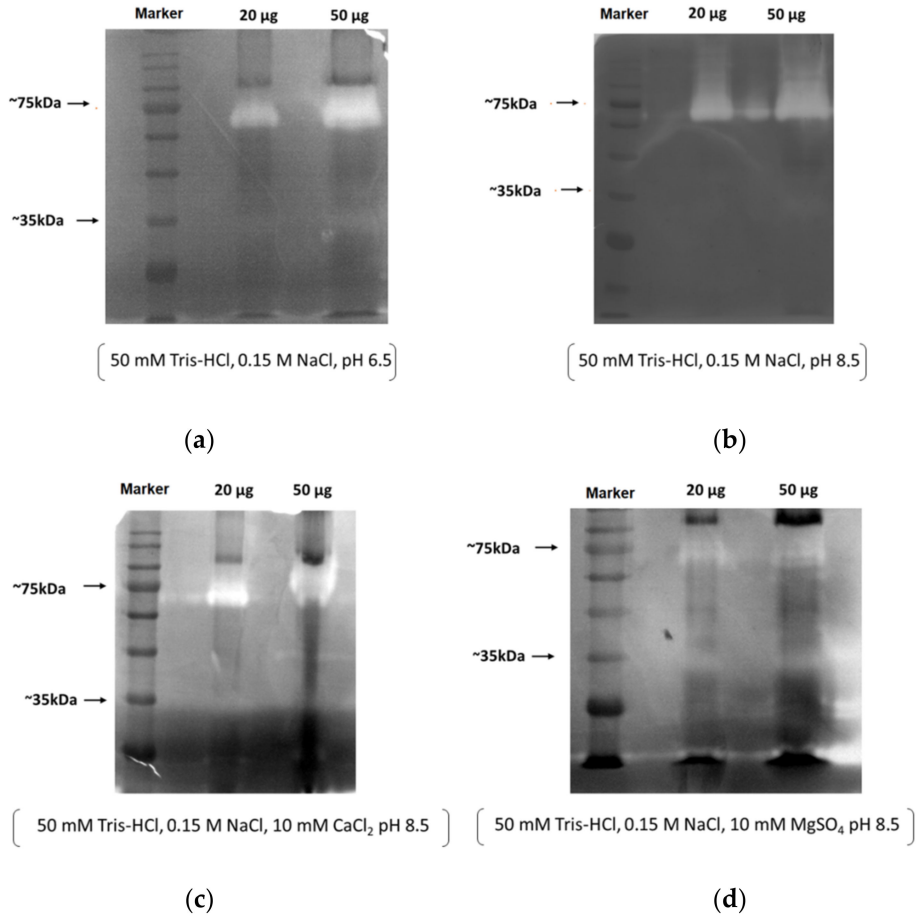


Figure 4. Zymographs of the *A. platensis* lysate with casein as a substrate and different buffers: (**a**) 50 mM Tris-HCl, 0.15 M NaCl, pH 6.5, (**b**) 50 mM Tris-HCl, 0.15 M NaCl, pH 8.5, (**c**) 50 mM Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, pH 8.5, (**d**) 50 mM Tris-HCl, 0.15 M NaCl, 10 mM $MgSO_4$, pH 8.5.

3.2. Development of an Aqueous Two-Phase System (ATPS) for the Purification of A. platensis Proteolytic Content

A mild method for the purification of the proteolytic enzymes of *A. platensis* lysate was developed using a PEG/phosphate aqueous two-phase system (ATPS). PEG/phosphate ATPS is a low-cost and rapid method with the potential for large-scale applications [81,82]. The initial conditions of the ATPS were based on previously gained experience [81,82]. The parameters that affect the purifying efficiency of the ATPS toward the desired proteins were adjusted and evaluated in order to establish the optimal system conditions. The evaluated parameters included the molecular weight (1500 or 3300 Da) of PEG, the pH (6.5–8.5) of the aqueous phase (phosphate buffer) and the salt concentration (NaCl, 0–10% *w/w*) in the system [69]. The results are listed in Table 1. As we can observe, the optimal conditions for the separation of proteolytic activity correspond to: 27.4% (*w/w*) PEG 1500 (stock solution 80% (*w/w*)), 32.7% (*w/w*) phosphate pH 7.0 with a concentration of 10% (*w/v*) NaCl, 30% (*w/w*) ddH_2O, 10% (*w/w*) *A. platensis* extract (protein concentration up to 1.7 mg/mL). The separation of proteins in the ATPS is accomplished at relatively low protein concentrations (e.g., 1.7 mg/mL). This concentration limit depends on each protein and its properties [83]. The distribution coefficient (K < 1) of the optimal system (Table 1) indicates the accumulation of the majority of the proteolytic activity at the bottom phase, resulting in high purification (12.86-fold) and efficiency (99.4%). Phycocyanin, which is found in high concentration in *Arthrospira platensis* and provides its blue-green color [39,40],

was distributed at the upper PEG phase of the system as observed by its blue coloration (Figure 5).

Table 1. ATPS experimental conditions (1–9) and obtained results of the partition and purification of the proteolytic activity of *A. platensis* lysate.

		1	2	3	4	5	6	7	8	9
MW_{PEG} (g/mol)		1500	3300	1500	1500	1500	1500	1500	1500	1500
C_{PEG} (*w*/*w*) (%)		27.4	33.8	27.4	27.4	27.4	27.4	27.4	27.4	27.4
pH		7	7	6.5	7.5	8	8.5	7	7	7
$C_{phosphate}$ (*w*/*w*) (%)		32.7	36.2	32.7	32.7	32.7	32.7	32.7	32.7	32.7
% (*w*/*w*) NaCl		0	0	0	0	0	0	0.3	0.7	10
K		0.014	0.097	1.875	0.224	2.5	-	0.026	0.034	0.005
K_p		4.597	2.157	1.979	2.317	2.335	2.495	2.159	2.638	1.848
K/K_p		0.003	0.045	0.947	0.097	1.071	-	0.012	0.013	0.003
Top phase	Specific activity (U/mg)	0.054	0.495	0.240	0.437	1.105	1.053	0.306	0.329	0.090
	Purification	0.024	0.223	0.086	0.157	0.397	0.378	0.131	0.139	0.038
	Yield(%)	1.18	13.14	71.95	16.97	69.46	100	2.89	3.75	0.61
Bottom phase	Specific activity (U/mg)	18.031	11.028	0.256	4.561	1.032	0	25.289	25.488	31.084
	Purification	8.152	4.984	0.092	1.637	0.37	0	10.785	10.543	12.857
	Yield (%)	98.82	86.86	28.05	83.03	30.54	0	97.11	96.26	99.40

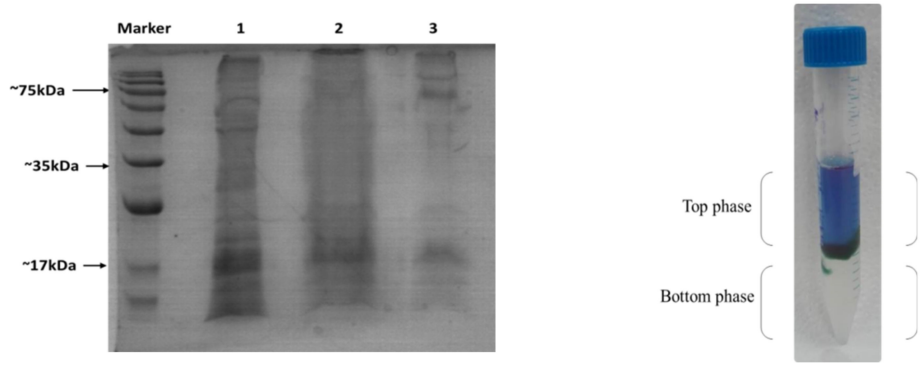


Figure 5. (**a**) SDS-PAGE of *A. platensis* lysate (1:50 µg of protein), bottom phase of ATPS (2:50 µg of protein) and bottom phase of ATPS after dialysis (3:12.5 µg of protein). (**b**) Top and bottom phases of ATPS (27.4% (*w*/*w*) PEG1, 500, 32.7% (*w*/*w*) phosphate pH 7 with a concentration of 10% (*w*/*v*) NaCl, 30% (*w*/*w*) ddH2O, 10% (*w*/*w*) *A. platensis* lysate (protein concentration 1.7 mg/mL)).

The bottom phase of the ATPS was evaluated by SDS-PAGE, and the molecular masses of the proteins are shown in Figure 5a. A main protein zone between 63 and 75 kDa and some other minor zones were clearly visible (Figure 5a, lane 3). Zymography of a bottom-phase sample allowed the identification of two protein zones with high proteolytic activity toward casein. The protein zone between 63 and 75 kDa and another with molecular weight around 35 kDa displayed proteolytic activity after incubation at pH 6.5 and 8.5 in the presence of Ca^{2+} (Figure 6).

Taking into account that the pH of the human skin is within the range of 5.2 to 5.8, a more targeted evaluation was elaborated for assessing the effect of pH on the proteolytic activity of the ATPS bottom phase. The effect of pH was determined in the range of 3.6 to 11.5 using the most effectively hydrolyzed substrate, azocasein. Figure 7 illustrates that the ATPS bottom phase displayed significant proteolytic activity in the acidic pH range (pH 3.6–6.0).

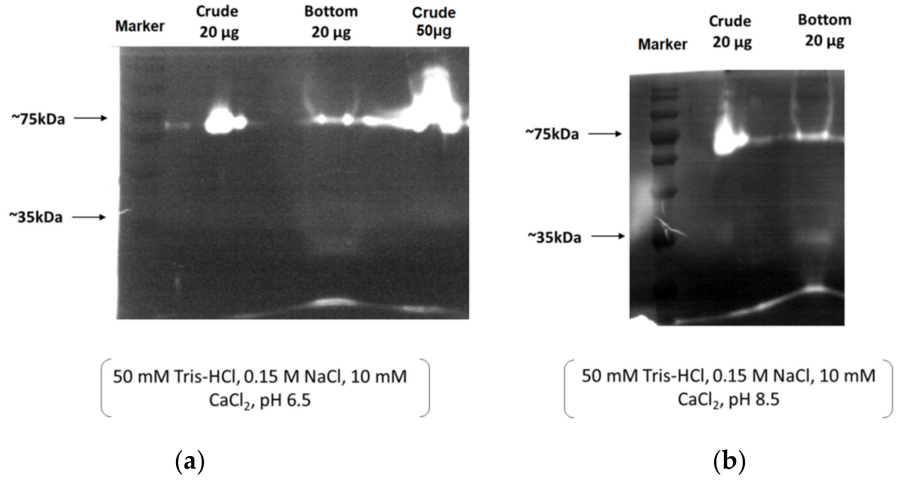


Figure 6. Zymography of the *A. platensis* lysate and the bottom phase of ATPS with different incubation buffers: (**a**) 50 mM Tris-HCl, 0.15 M NaCl, 10 mM CaCl2, pH 6.5, and (**b**) 50 mM Tris-HCl, 0.15 M NaCl, 10 mM CaCl2, pH 8.5.

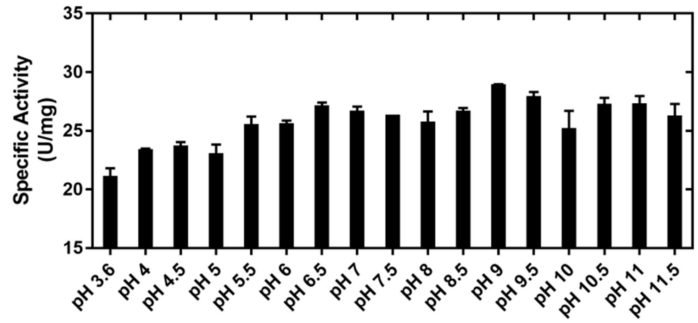
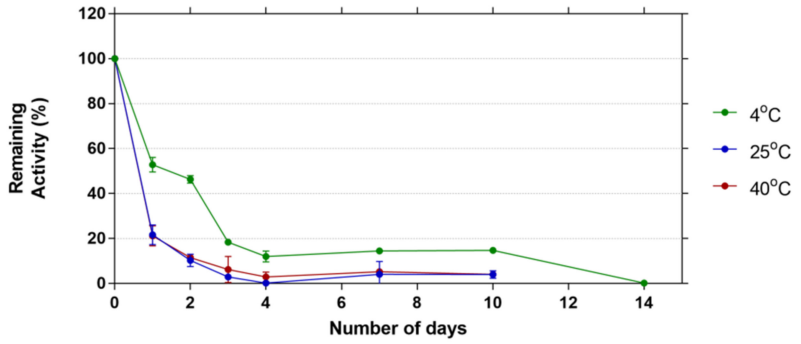
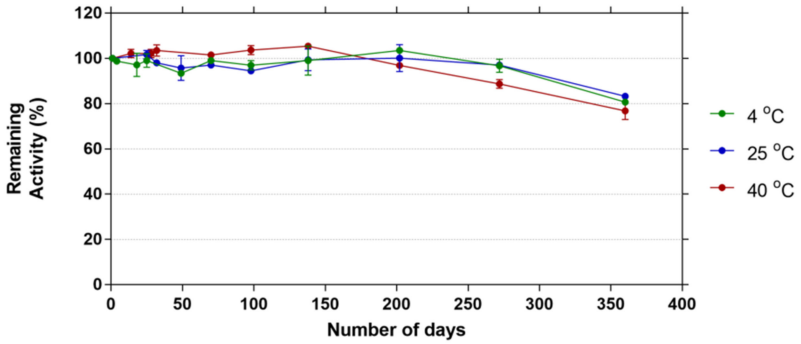


Figure 7. Specific activity (U/mg) of the bottom phase of ATPS in pH range of 3.6 to 11.5 using 2% (*w*/*v*) azocasein as substrate. The data represent the mean ± SD (N = 3).

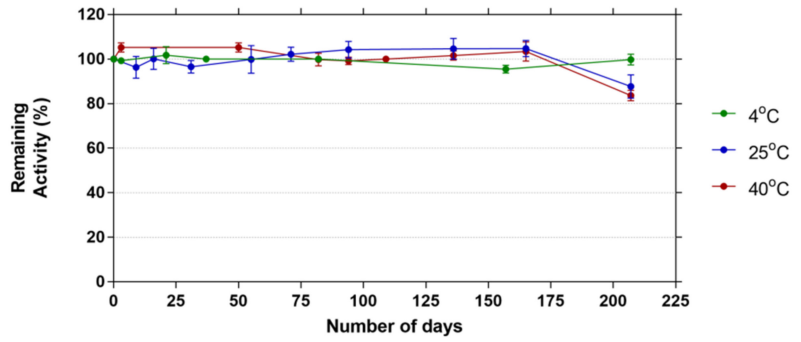
The stability of the proteolytic activity of *A. platensis* lysate and of the ATPS bottom phase was investigated at temperatures of 4 °C, 25 °C and 40 °C. The proteolytic activity was determined with (2% *w*/*v*) azocasein as a substrate. The results show that the *A. platensis* lysate exhibited approximately 80% reduction in proteolytic activity after one-day storage at 25 °C and 40 °C, and after three days, no detectable activity was measured. However, the lysate at 4 °C showed a more gradual decrease in its proteolytic activity, which was completely lost after fourteen days (Figure 8a). Interestingly, the ATPS bottom-phase samples exhibited extremely high stability, since it remained nearly stable even after one-year storage in all the selected temperatures (Figure 8b). These samples contained a large salt concentration which was expected to favor the enzyme stability [84]. The remaining activity after 360 days was approximately 80% for the samples incubated at 4 and 25 °C, with a slightly higher decrease after 200 days of incubation at 40 °C.



(**a**)



(**b**)



(**c**)

Figure 8. Remaining activity (%) after storage at different temperatures (4 °C, 25 °C and 40 °C) of (**a**) the *A. platensis* lysate, (**b**) the bottom phase of ATPS and (**c**) the hydrogel that was formulated with *A. platensis* lysate. 100% proteolytic activity refers to the measurement at Day 0, using azocasein as substrate. Measurements were performed in triplicate, and the data present the mean ± SD (N = 3).

3.3. Development of a Hydrogel Formulation with Proteolytic Activity

A hydrogel was developed with the *A. platensis* lysate (92.8% (*w/v*)) as the main ingredient. Hydrogels appeared in the late 1950s and consisted of cross-linked networks that can retain significant amounts of water [85].

The formulated hydrogel contained 3.4% (*w/v*) of the moisturizing and antimicrobial agent Dermosoft® OMP (phenylpropanediol, 1,2-octanediol, phenylpropanol) and 3.8% (*w/v*) of Lecigel™ (sodium acrylates copolymer and lecithin), which induces the coagulation of the hydrogel. The properties of hydrogels can be manipulated and regulated by the use of suitable additives and materials such as lecithin [86,87]. Lecithin is an amphiphilic phospholipid mixture primarily containing distearoylphosphatidylcholine, which in water systems can self-assemble into an array of liquid–crystalline structures. Lecithin can

affect the structure, functionality and mechanical properties of hydrogels [86,87]. These commercial ingredients are suitable for dermal use; therefore, they were selected in order to examine the potential application of *A. platensis* lysate in a cosmetic formulation that would conserve its proteolytic activity. The developed formulation did not result in any significant activity loss after the incorporation of the lysate in the hydrogel (Table 2). A control hydrogel that did not contain the microalgal lysate was also tested (92.8% (*w/v*) phosphate buffer), with no detectable proteolytic activity.

Table 2. Specific activity (U/mg) of the *A. platensis* lysate using azocasein as a substrate, before and after the hydrogel formulation. The data represent the mean ± SD (N = 3).

Samples	Specific Activity (U/mg)
A. platensis extract	0.262 ± 0.016
Hydrogel formulation	0.255 ± 0.007

The stability of the hydrogel-entrapped proteolytic activity was investigated at three temperatures, 4 °C, 25 °C and 40 °C, and the results are illustrated in Figure 8c. The results show that the entrapment of the lysate in the hydrogel leads to a dramatic improvement in its stability at all tested temperatures (4 °C, 25 °C and 40 °C). In particular, at 4 °C, the enzyme exhibited the highest stability and retained nearly all of its proteolytic activity after 207 days of incubation. It is noteworthy that the activity was retained even after 456 days of incubation at 4 °C (93.85 ± 1.28% residual activity, data not shown in Figure 8c). At 40 °C, the residual activity was 83.7 ± 2.4% after 207 days of incubation, while at 25 °C, it was 94.4 ± 1.4% (Figure 7c). Therefore, the hydrogel formulation significantly improved the stability of the proteolytic activity of *A. platensis* lysate which was completely inactivated after fourteen days at 4 °C or even earlier at 25 °C and 40 °C (Figure 8a).

The application of proteolytic enzymes in dermatology is being investigated for wound healing and removal of necrotic tissues from wounds and burns [51,52]. In addition, the incorporation of proteolytic enzymes in cosmetology has been proposed in cleansers and moisturizers in order to restore and maintain the skin barrier as well as to reduce inflammation due to trauma [56]. Proteases mainly aim to promote exfoliation of the surface epidermal layer and ultimately increase the absorption of water or other ingredients [60,61], which in conjunction with the *A. platensis* innate bioactive ingredients [22,26–29] may be a promising base for the development of innovative and effective cosmeceutical products. Considering the wide available biodiversity of microalgae, it is conceivable that several microalgae species with potential bioactive properties can also be used and exploited for developing similar applications in the cosmetics industry [88–90].

Further studies using skin cell lines are needed in order to evaluate the cytotoxicity and the appropriate concentration of the *A. platensis* entrapped-proteolytic enzymes for future dermal use.

4. Conclusions

In this work, the proteolytic activity of *Arthrospira platensis* lysate was investigated. The results indicate the presence of at least two different proteolytic enzymes with different molecular masses and kinetic properties. The proteolytic activity was partially purified using a mild one-step rapid protocol, based on a PEG/phosphate ATPS. The proteolytic activity of the lysate was entrapped in a hydrogel, leading to a stable formulation that provided enhanced enzyme stability at 4, 25 and 40 °C, compared to the free enzyme extract. This highly stable hydrogel entails significant potential in cosmetology as a skin exfoliating agent. Moreover, the use of the proteolytic enzymes of *A. platensis* is consistent with the growing demand for safe and environmentally friendly products from sustainable resources.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics9050106/s1.

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