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Pulsed electric field (PEF)-assisted protein recovery from *Chlorella vulgaris* is mediated by an enzymatic process after cell death

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A B S T R A C T

Pulsed electric field (PEF) treatment has so far been considered as ineffective for protein extraction, but the parameters that influence protein extraction efficiency have not been identified fully. Using *Chlorella vulgaris* in this study, we show that up to half of the proteins present in the cell can be extracted via PEF treatment and an incubation step for up to 24 h. The extraction efficiency is highly influenced by biomass concentration during the incubation step and the incubation temperature: in our experiments we were able to extract half of the proteins present in the cells at dilute concentrations (5 mg·ml^{-1}), but this efficiency decreases with increasing biomass concentration, indicating that diffusion gradients play a role in the protein release. However, we also show that not only diffusion plays a role, but also a biological process that requires suitable conditions. We observed that PEF treated cells undergo programmed cell death, indicated by DNA laddering. Furthermore cold and hot temperatures as well as protease inhibition impair the release of proteins. In Western blots we can observe that some proteins are not released when the activity of proteases is blocked. We therefore conclude that protein release is facilitated by an enzyme-driven process that occurs after PEF-triggered cell death, and that this process is prone to extreme conditions.

1. Introduction

Due to the growing demand for resources and competition of energy and crop plants for arable land, microalgae are currently being discussed as a novel source of food, feed and high-value compounds such as pigments, protein, lipids and pharmaceuticals since they can be cultivated in non-arable areas [1]. Some microalgae such as *Chlorella vulgaris* and the cyanobacterium *Spirulina platensis* are already certified for food and feed applications as they are generally regarded as safe ("GRAS" status) and are also being marketed as "superfood" already [2].

The focus in this study lies on protein extraction as they could potentially be used for food and feed applications. Various extraction methods for proteins are being investigated during the last years, most of them being mechanical methods like high-pressure homogenization (HPH) [3] or bead-milling [4]. While these methods are reported to be quite efficient in terms of protein extraction efficiency, they create cell debris that is hard to separate in industrial processes [5]. As an alternative cell disruption method, pulsed-electric fields (PEF) are also being investigated and have already been applied to microalgae [6–9]. The consequence of PEF treatment is membrane electroporation [10]. PEF treatment has already been shown to increase extraction yields for lipid extraction [11,12], but the utilization of PEF for protein extraction has not been fully investigated so far. There are reports confirming that protein extraction is conceptually possible [7], although in some studies only moderate success has been reported as of now [6,13]. However, investigating PEF-assisted extraction is appealing because it is potentially possible to get a higher degree of extraction selectivity towards certain valuable fractions such as proteins and lipids, whereas mechanical methods such as HPH scramble these fractions together, emulsifying lipids and proteins as reported in [3] for instance. This means that PEF allows for fractionation of the biomass into different phases (e.g. lipids, proteins).

Among the myriad of extraction methods [14,15] are also chemical and enzymatic extraction approaches. In comparison with these, PEF would have the advantage that no chemical additives would be necessary besides the adjustment of conductivity which can be accomplished with salts, providing a non-toxic extraction method that could provide food-grade proteins. There are attempts to combine PEF as a pretreatment to mechanical methods such as HPH, however no combinatory effect can be seen with PEF and HPH [5]. When optimizing PEF extraction parameters for proteins, it could be possible to establish a "wet" processing cascade in which water-soluble proteins are extracted first and then sequentially lipids could be extracted in a "wet" extraction as reported by [11,12], all this without the need to dry the biomass first. Investigating extraction parameters for various compounds might enable a biorefinery cascade for microalgae biomass as it is being applied to other biomasses already [16].

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Coustets et al. [7] have reported that protein extraction after PEF treatment requires an incubation step. The aim of this study is to determine which processes take place upon PEF treatment and which incubation parameters have an impact on protein release after PEF treatment, as it could be possible to optimize the incubation in order to obtain higher protein extraction yields.

2. Material and methods

2.1. Algae cultivation and sample preparation

C. vulgaris (strain 211-12 purchased from the SAG University of Göttingen) cells were grown in 400 ml 1× TAP medium (0.02 M TRIS, 0.001 potassium phosphate buffer, 1× TAP salts [17], 1× Hutner's trace elements [18], 0.001% acetic acid, pH 7.0) in 1000 ml flasks. They were inoculated at an OD of 0.1 and left to grow on a shaker at constant agitation, 23 °C and 60 $\mu E \cdot s^{-1} \cdot m^{-2}$ of light illumination with fluorescent lamps. They were grown for 7 d until they reach an OD around 2 and then concentrated by centrifugation (10,000 ×*g*, 2 min). At this point, the cells are in stationary phase of their growth. The biomass concentration and dry weight was determined gravimetrically using a precision scale after drying the suspension in aluminum cups at 80 °C for approximately 2 h. The concentration was then adjusted to a desired concentration, typically 5 or 10 $g \cdot l^{-1}$, using leftover medium from the centrifugation steps. Under these conditions, the algae have an average protein content of 45% related to dry biomass ($\%_{DBM}$).

2.2. HPH cell disruption method

For the HPH method, the cell suspension was passed through an Avestin EmulsiFlex high pressure homogenizer (2 kbar, 5 passes) cooled with ice. This method proved to extract all proteins in our case. A reference for this method can be found in [3]. The cell debris was separated by centrifugation (10,000 ×*g*, 10 min, 4 °C) afterwards.

2.3. Pulsed electric field treatment

Depending on the sample volume and sample size the algae suspension, typically with a biomass concentration of 10 $g \cdot l^{-1}$, was pulsed either in a continuous flow chamber using silicon tubings and a peristaltic pump (MS-4/12-100 ISMATEC, Cole-Parmer GmbH, Wertheim, Germany) or in 500 µl electroporation cuvettes with a gap distance of 2 mm (Electroporation Cuvettes Plus, BTX Harvard Apparatus, Holliston MA, USA). The continuous flow chamber had a volume of approximately 500 µl enclosed by two planar electrodes with 2 mm gap distance as described in [9]. The flow rate for continuous flow mode was set to 3 $ml \cdot min^{-1}$ and the pulse frequency was set to 4.5 Hz. For both methods, the rectangular pulses had a field strength of 40 $kV \cdot cm^{-1}$ and a duration of 1 µs. The resulting specific treatment energy was 150 $J \cdot g^{-1}$. In the cuvettes, the samples received 47 pulses with a pulse frequency of 1 Hz to mitigate heating. The algae suspensions usually had an initial temperature of 21 °C and never exceeded 38 °C during the pulse treatment in either configuration. The field strength of 40 $kV \cdot cm^{-1}$ and the specific energy of 150 $J \cdot g^{-1}$ was used because based on empirical data, these parameters ensure that basically all cells are irreversibly electroporated [8,9,19] and thus were also applied for other works from this group [11,12].

2.4. Post-PEF incubation

Protein extraction after PEF treatment requires an incubation step [7]. After the pulsing, the algae suspension was diluted with water to 5 $mg \cdot ml^{-1}$ and incubated for a certain time and at different temperatures to test the influence of diffusion. For the biomass-concentration dependence experiment, the algae suspension was adjusted to a conductivity of 2 $mS \cdot cm^{-1}$ using NaCl and pulsed in cuvettes with 2 mm gap distances at 25, 20, 15, 10 and 5 $mg \cdot ml^{-1}$. Afterwards these suspensions were diluted with an equal amount of water (in this case 500 µl deionized water) and incubated for 24 h at room temperature. After incubation the cells were spinned down (10,000 ×*g*, 10 min) and the protein content/released protein in the supernatant was quantified with the BioRad DC-assay Kit. This incubation step was modulated by incubating the samples at alkaline conditions with 0.1 M NaOH and/or protease inhibitor (c0mplete Plus by Roche) to test whether pH has an influence on the protein extraction efficiency and whether enzymes such as proteases are involved in the process.

2.5. Protein quantification

Protein content was determined using the Merck Direct Detect infrared spectrometer as well as the BioRad DC-assay, which is essentially a detergent-compatible Lowry assay. For the infrared spectrometry, cells were diluted to a concentration within a range of 2.5–5 $mg \cdot ml^{-1}$ and 2 µl of the suspension was then measured with the device. The supernatants and extracts obtained via HPH and PEF were quantified using the BioRad DC-assay Kit against a BSA calibration ranging from 0.2 to 2 $mg \cdot ml^{-1}$ according to the manufacturer's protocol. In both cases, the resulting biomass concentration was divided by the biomass concentration to calculate the protein content in $\%_{DBM}$.

2.6. Evans blue staining

Evans blue staining was used to check for membrane integrity after PEF treatment. Briefly, 500 µl of a cell suspension was pulsed in an electroporation cuvette (2 mm gap) and then an equal volume of a 5% Evans blue solution (MW: 961 Da) was added at certain time points (immediately, 1, 6 and 24 h post-PEF). The sample was mixed with the pipette and then incubated for 10 min, then transferred to a 2 ml microfuge tube, spinned down briefly (10,000 ×*g*, 2 min) and washed 3 times with 2 ml deionized water. Non-pulsed cells served as a control and were treated the same way. The samples were analysed by microscopy (Zeiss Axioplan 2) at 63× magnification. Cells were counted using ImageJ and the permeabilization efficiency was calculated by dividing the number of stained cells by the number of total cells multiplied by 100.

2.7. DNA extraction for investigation of DNA laddering

For the DNA extraction, the PEF treated algae were spinned down (10,000 ×*g*, 10 min) in 2 ml microfuge tubes and separated into pellet and supernatant. Afterwards, these samples were frozen in liquid nitrogen and freeze-dried (CHRIST, Alpha 1-4 LDplus). The dried algae pellets were ground by inserting a 4 mm steel bead into the reaction tubes, cooling the tubes with the beads in liquid nitrogen and then agitating the tube at 30 Hz for 30 s (Retsch TissueLyser). The DNA was afterwards extracted from the ground material using the Invisorb Spin Plant Mini Kit according to the manufacturer's protocol and visualized on a 1,5% agarose gel in 0.5% TAE (20 mM TRIS, 10 mM acetic acid, 0.5 mM EDTA) stained with 5 µl SYBR Safe DNA gel stain per 100 ml of agarose.

2.8. SDS-PAGE and Western blot detection of organelle-specific proteins

The extracts obtained by PEF treatment were mixed with 4× Lämmli buffer (200 mM TRIS-HCl, 8% (w/v) SDS, 40% (v/v) glycerol, 4% (v/v) β-mercaptoethanol, 0.8% (w/v) bromphenol blue) in a ratio of 4:1 (e.g. 300 µl extract + 100 µl 4× Lämmli) and then heated to 95 °C for 15 min [20]. The proteins were separated on a 12% polyacrylamide gel via SDS-PAGE (BioRad). The gels can then be stained by Coomassie staining [21] or subsequently blotted onto a nitrocellulose membrane (Roti-NC by Carl Roth). After blocking with cream liquor (Baileys), the membrane was washed and antibodies were applied for

organelle-specific proteins. The primary antibodies used in this study are directed against RuBisCo for chloroplast (Abcam, ab226002), Histone H3 for nucleus (Abcam, ab1791), Actin for cytosol (Agrisera, AS132640) and Cytochrome c oxidase subunit II (COXII) for mitochondria (Agrisera, AS04053A). These are all rabbit polyclonal antibodies, a goat anti-rabbit secondary antibody coupled to horseradish peroxidase (HRP) served as secondary antibody for detection. The signals were developed colorimetrically using 1-Step TMB-Blotting Substrate Solution (Thermo Fischer Scientific).

2.9. Statistical analysis & replication

Each experiment was repeated at least 3 times in triplicates each time. The graphs show average values of 3 independent experiments with their standard deviation. Statistical significance was determined by paired student's *t*-test when applicable. Gels, microscopy, Western blots and DNA extraction were also done at least three times, the most representative were chosen for publication.

3. Results

3.1. Evaluation of cell death after PEF treatment

In order to confirm that membrane integrity of *C. vulgaris* cells is in fact affected by the PEF treatment they were stained with Evans blue, a dye that does not penetrate intact cells (Fig. 1, Control) but can accumulate in permeabilized cells (Fig. 1, PEF treated). Evans blue is able to penetrate cells right after the PEF treatment and can also do so at any point after the treatment (i.e. 1, 6, 24 h). This shows that the cells are immediately and irreversibly permeabilized by the PEF treatment under these parameters. The staining efficiency was determined by cell counting and is around 99,92%.

Evans blue is used as an indicator of cell death [22]. Because it is often neglected that cells can in principle recover and repair their membrane after uptake of the dye, we also extracted DNA of pulsed *C. vulgaris* and investigated for DNA laddering (Fig. 2) to back up the assumption that PEF treatment under these parameters is lethal to *C. vulgaris*. The genomic DNA appears as one clear band in the pellet of the control (Fig. 2, 6 h CTRL). DNA extracts from the pellets of PEF-treated samples exhibit DNA laddering that progresses over time (Fig. 2, 1–6 h P). DNA fragments can also be detected in the supernatants (Fig. 2, 4 h S and 6 h S), indicating that the cells are also leaking their DNA material into their surrounding post-PEF. The samples processed by the HPH method only show a slight smear.

3.2. Effect of biomass concentration during incubation on protein extraction yield

The protein extraction efficiency in dependence of biomass concentration was determined by serial dilution of the algae suspension to the according biomass concentrations (2.5, 5, 7.5, 10, 12.5 $mg \cdot ml^{-1}$) and subsequent pulse treatment in cuvettes. Our results show that within a range of 2.5 to 12.5 $mg \cdot ml^{-1}$, the biomass concentration can decide between extracting half of the proteins present in the cell and one third of the proteins:

At 2.5 $mg \cdot ml^{-1}$, we extract 22%$_{DBM}$ of protein, which is - given that the algae have an average protein content of 45%$_{DBM}$ - roughly half of the proteins in the cell.

At 12.5 $mg \cdot ml^{-1}$, we extract 15%$_{DBM}$ of protein, which is only one third of the proteins.

3.3. Impact of incubation temperature on protein recovery

To determine the influence of incubation temperature on the protein yield after PEF treatment, 2 ml aliquots of PEF treated samples with a concentration of 5 $mg \cdot ml^{-1}$ were incubated at different temperatures 4, 23, 30, 40 and 50 °C. The extraction kinetic was plotted by quantifying the released protein in the supernatant after 1, 2, 4, 6 and 24 h. The extraction kinetics vary between the different temperatures. The extraction efficiency has an optimum around 30 °C. The temperatures 4 and 50 °C however show a restrictive effect on the extraction. They are also significantly different to 23, 30 and 40 °C. The differences between 23, 30 and 40 °C are statistically insignificant. For the high and low temperatures the extraction efficiency does not exceed 12%$_{DBM}$ after 24 h, far below the protein concentrations obtained at 23, 30 and 40 °C. It is apparent that the extraction as we perform it has a temperature optimum around 30 °C, with the end points of the 23, 30 and 40 °C batches all clustering together.

3.4. Impact of highly alkaline pH and protease inhibitor on PEF extraction efficiency

The results from the temperature variation suggested that a process that goes beyond diffusion, possibly an enzyme-driven one, could

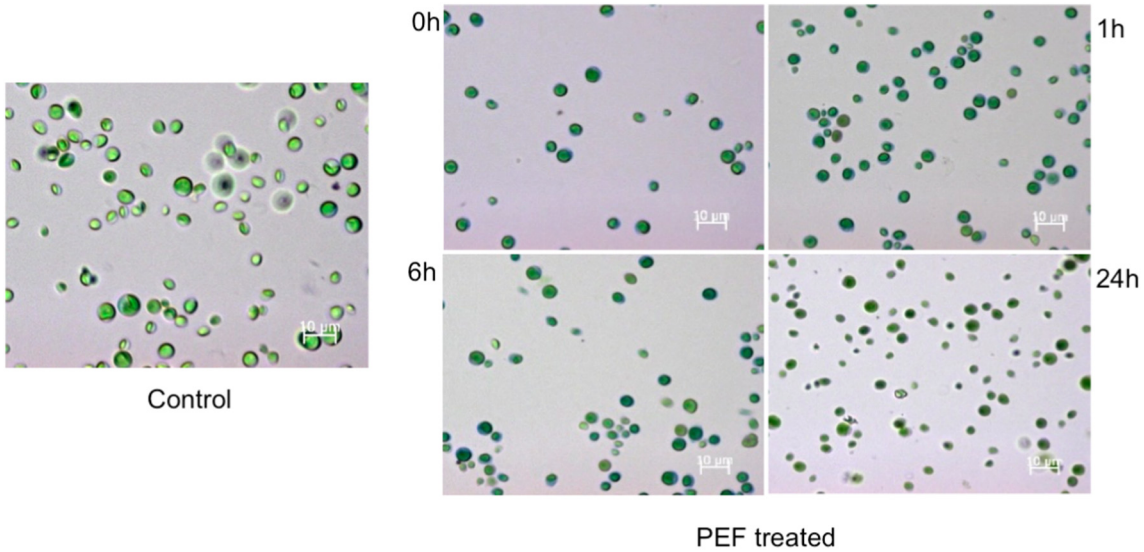

Fig. 1. Evans blue staining of *C. vulgaris* after PEF treatment. *C. vulgaris* suspensions were PEF-treated and afterwards stained with Evans blue dye at various time points. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

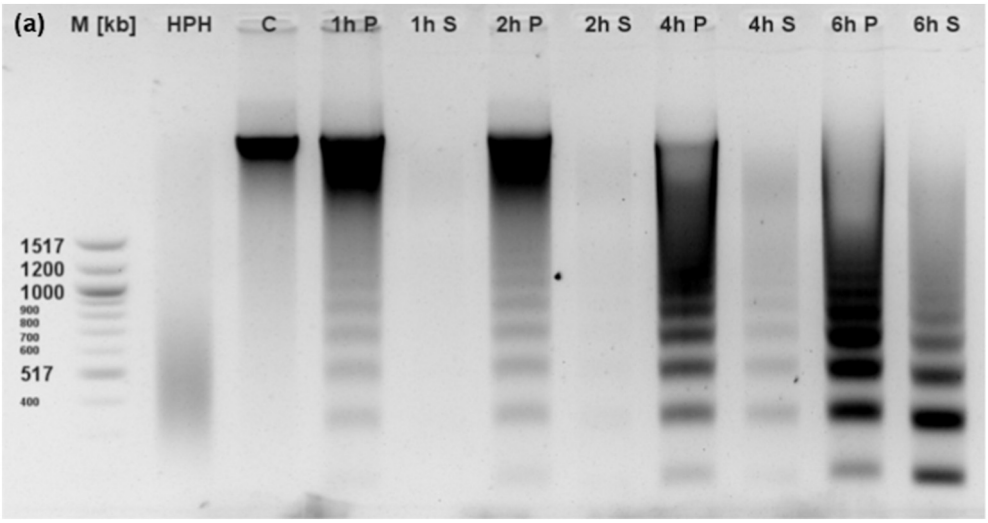

Fig. 2. Analysis of DNA-laddering after PEF treatment. Genomic DNA of PEF treated *C. vulgaris* was extracted from freeze-dried pellets (P) and supernatants (S) and visualized on a 1,5% TAE agarose gel.

influence protein release, because protein release seems to work best in a physiological temperature range but not at extreme temperatures. We therefore also investigated the effect of alkaline pH and the impact of protease inhibitor on the extraction yield because these could interfere with the protein release if this process is supported by proteases. Extraction in 0.1 M NaOH (pH 13) actually yields less protein than extraction in medium (Fig. 5, PEF Medium and PEF NaOH). However, the 0.1 M NaOH already has a disruptive effect on the control cells per se (Fig. 5, CTRL NaOH). When cells were incubated with protease inhibitor, the protein release is decreased even further. An additive effect of 0.1 M NaOH and protease inhibitor cannot be seen, the differences between extractions in NaOH and NaOH combined with protease inhibitor are insignificant, whereas other differences are significant ($p < 0.05$).

3.5. Visualization of extracted proteins by SDS-PAGE and Coomassie staining

To see differences between mechanical extraction methods like HPH and PEF treatment, we loaded samples of PEF extracts from a time course experiment (incubated at 23 °C; see Section 3.3) onto a 12% polyacrylamide gel together with an extract obtained by HPH. The extracts in the PEF time course show bands that get more pronounced and intense over time (Fig. 6, PEF treated 1–24 h). The HPH extract (Fig. 6, HPH) represents a total protein extract to which there are only minor differences when compared with the PEF extracts, except for the signal intensity and the very pronounced signal at 25 kDa.

3.6. Localization of PEF extracted proteins from C. vulgaris

Western blot detection of organelle-specific proteins shows that proteins of all the tested organelles can be detected in the supernatant of the cells treated with PEF. For RuBisCO, Histone H3 and Actin a signal can be detected already after 1 h of incubation post-PEF treatment (Fig. 7 RuBisCo, Histone H3 and Actin). The signal for COXII shows up after an extended period of time 6 h after the PEF treatment (Fig. 7, COXII). The signals for actin and COXII do not match the molecular sizes predicted by the manufacturers of the antibodies, so it is questionable whether the signals really correspond to the according proteins, but in the PEF extracts there is only one band visible, indicating some specificity of the antibodies. Overall, the Western blots show that the signals for the proteins intensify over time, confirming the time-dependent manner of protein release after PEF. The band for Histone H3 exhibits a size shift and seemingly becomes smaller over time. It has to be explicitly pointed out that in Western blots of samples incubated with protease inhibitor we don't observe the size shift in the histone band and the COXII band is not detectable. The signals for the other proteins are slightly weaker under the influence of protease inhibitor (data not shown).

4. Discussion

Early on in our experiments, we initially observed a fluctuation in extraction efficiency due to not normalizing the biomass concentration and/or temperature during extraction. We therefore investigated the influence of biomass concentration and found that extraction yield decreases with increasing biomass concentrations (Fig. 3). In our range between 2.5 and 12.5 $mg \cdot ml^{-1}$ we could observe that even this range can decide between extracting half of the proteins or one third of the proteins present in the cells. This can explain the lower extraction values reported by other studies in which higher biomass concentrations are used; [13] for instance used 25 $mg \cdot ml^{-1}$. One reason for this phenomenon could be that the biomass concentration influences the diffusion gradient of the proteins from the inside to the outside of the cells. This circumstance will be a challenge for upscaling in industrial applications, it needs to be elucidated how to maintain a good extraction efficiency at high biomass concentrations. We then decided to normalize our biomass concentration (5 $mg \cdot ml^{-1}$) and proceeded to check for the influence of temperature on protein release. The extraction works well in a range between 20 °C and 40 °C. However, at 4 and 50 °C respectively, the maximum yield over the 24 h period is around 12%, half of what can be obtained at 20, 30 or 40 °C (Fig. 4). This indicated to us that - besides diffusion - there might be some enzyme-driven process involved in the protein release, most likely one that occurs after cell death and that is inhibited by non-physiological temperatures. We therefore checked for cell death markers like DNA laddering and Evans blue staining. Evans blue staining revealed that the cells are all permeabilized immediately after the treatment and stay permeable, showing that our treatment is irreversible (Fig. 1). Irreversible permeabilization of cell membranes is usually a hallmark for cell death [23]. To back this up we also looked at the DNA level and observed that DNA laddering starts within 1 h after PEF treatment and progresses over time, with the genomic DNA fragmentizing completely within 24 h (Fig. 2). This is usually seen as a hallmark for programmed cell death (PCD) [24], a controlled way of dying, but other publications report that DNA laddering also takes place in necrosis [25,26] which contrary

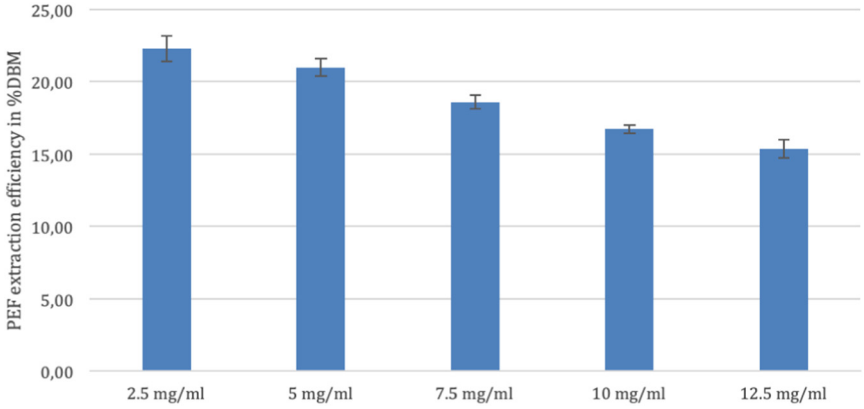

Fig. 3. Protein recovery efficiency from *C. vulgaris* in dependence of biomass concentration. *C. vulgaris* suspensions of various concentrations were pulsed (40 kV·cm^{-1}, 150 J·g$^{-1}_{suspension}$), diluted and incubated for 24 h at room temperature. Data are shown as average with standard deviation, n = 3.

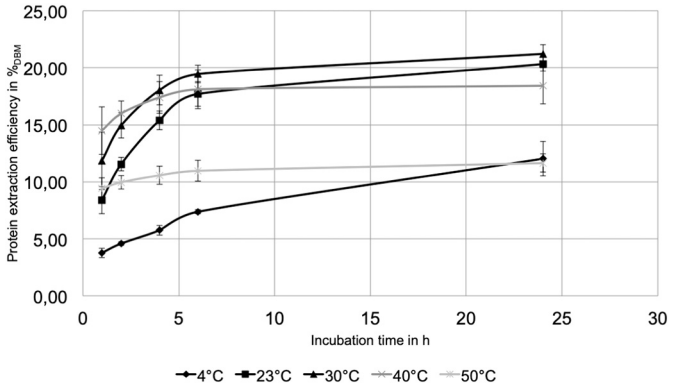

Fig. 4. Time course of protein extraction efficiency from *C. vulgaris* in dependence of extraction temperature. The algae suspension was pulsed (40 kV·cm^{-1}, 150 J·kg$^{-1}_{suspension}$) and then incubated at different temperatures. Data are shown as average with standard deviation, n = 3.

to PCD is an uncontrolled way of dying. The HPH processed samples don't show bands on the TAE gel because the high shearing forces of the HPH method degrade DNA. Either way the experiments so far suggested that there might be biological processes contributing to the protein release such as proteolytic activity of enzymes that help to cleave proteins and release parts of membrane and/or membrane-associated proteins into the environment. We therefore also modified extraction conditions in that we used high pH and also protease inhibitor to see the influence on these processes. We used 0.1 M NaOH to set the extraction

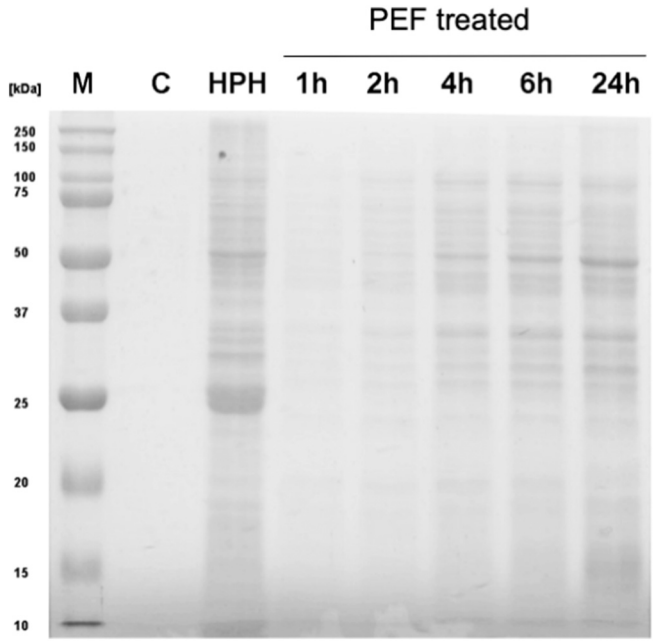

Fig. 6. Visualization of the protein extracts obtained by HPH and PEF treatment by SDS-PAGE and subsequent Coomassie staining. PEF samples of various timepoints and a HPH extract from a *C. vulgaris* suspension (5 mg·ml^{-1}) were loaded onto a 12% polyacrylamide gel and separated.

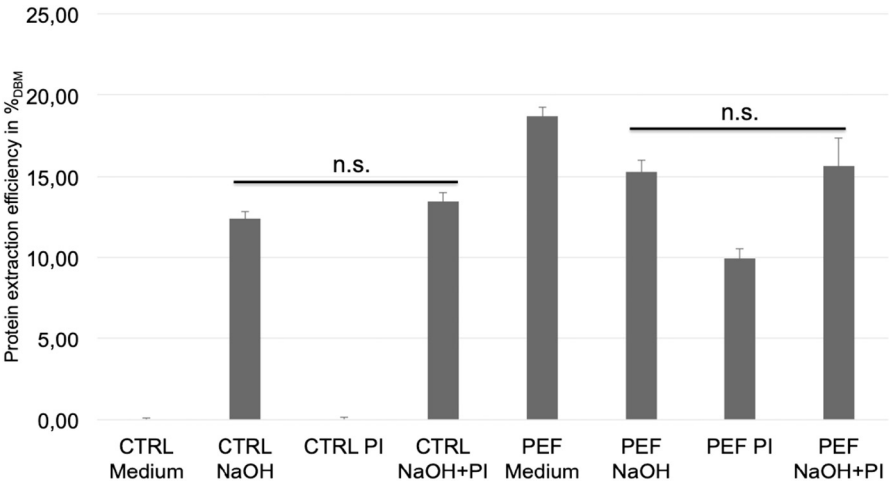

Fig. 5. Protein recovery efficiency from *C. vulgaris* under the influence of 0.1 M NaOH and protease inhibitor. The algae suspension (10 mg·ml^{-1}) was pulsed (40 kV·cm^{-1}, 150 J·g$^{-1}_{suspension}$) and afterwards incubated for 24 h at room temperature under the influence of 0.1 M NaOH and/or protease inhibitor. Data are shown as average with standard deviation, n = 3. CTRL = control, PEF = pulsed electric field treated, NaOH = sodium hydroxide, PI = protease inhibitor.

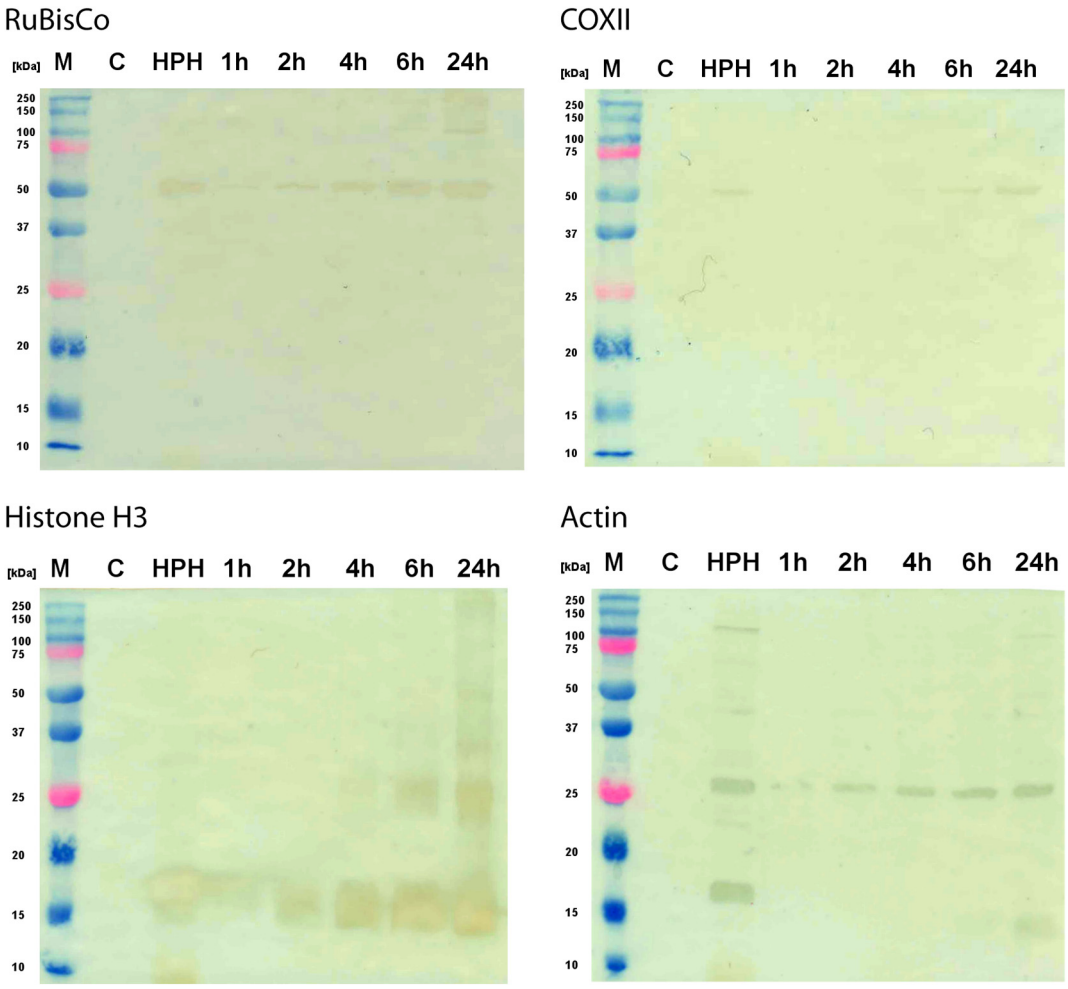

Fig. 7. Western blot detection of organelle-specific proteins. Organelle-specific proteins in the PEF extracts were detected after Western blot using antibodies directed against RuBisCo, COXII, Histone H3 and Actin.

pH to around 13 because publications such as the one by [3] suggest that a high pH (12 in their case) helps in solubilizing proteins. However, we observed a slightly decreased extraction yield when we used 0.1 M NaOH, most likely due to inhibition of enzymes by high pH. However, 0.1 M NaOH itself already has an effect on the cells alone as there is protein detectable in the supernatant after incubation of untreated cells in 0.1 M NaOH for 24 h due to its corrosive effect. Our hypothesis behind the protease inhibitor experiment was that 4 and 50 °C extraction temperature will also inhibit enzymes such as proteases that contribute to the release of some portion of the proteins present in the cell. If that is the case, protease inhibitor should exhibit the same or lower extraction yield as the extractions carried out at 4 and 50 °C without the corrosive effect, which is exactly what we can see: The extractions at 4 °C, 50 °C and the one at room temperature (23 °C) but with protease inhibitor all show an extraction yield of roughly 10%. The results overall suggest that proteins are released by a proteolytic activity after triggering cell death via PEF treatment, and that this activity can be disrupted by extreme conditions such as high and low temperatures (4 and 50 °C), pH and inhibitors. A very similar mode of action has also been described for yeast [29,30] and utilized to extract mannoproteins from baker's yeast [28] and pigments from red algae and other yeast species [27,29,30].

However, until this point we still did not have information about which parts of the cell and which organelles are actually affected by the PEF treatment, and which kind of proteins are extracted. The difference between the extracts obtained by HPH and PEF don't show obvious differences in the Coomassie-stained polyacrylamide gel (Fig. 6) beside the very pronounced band at 25 kDa in the HPH extract. It is very likely that this is chlorophyll *a*-b-binding protein, a largely abundant protein located in the thylakoid membranes of chloroplast [31,32]. The fact that this protein is not detected in the PEF extracts despite its abundance shows that there is a selectivity of PEF extraction towards soluble proteins, i.e. proteins that are not membrane-associated or transmembrane proteins. [7] have reported that they extract only cytosolic proteins with their extraction and pulse parameters. In order to investigate this further, we therefore chose to detect proteins characteristic for various organelles on Western blots to see whether organelles within the cytosol could also be affected by the PEF treatment. As the Western blots show we can detect proteins from mitochondria, chloroplast, nucleus and the cytosol. The band for actin does not have the predicted size as indicated by the antibody's manufacturer; we suspect that we are detecting an actin-related protein. However, actin-related proteins are mostly cytosolic as well, and the antibody generates one sharp and specific signal in the PEF extracts, thereby validating that proteins from the cytosol are indeed released. The band for COXII is only visible after a longer period of time, a signal is only detectable after 6 h compared to all other proteins that can already be detected after 1 h. COXII is a membrane-associated protein that needs to be processed by enzymes to release it into the surrounding (e.g. cleavage of membrane-anchors or transmembrane domains by intermembrane proteases). In a Western blot done with extracts including the protease inhibitor, we fail to detect COXII in a Western blot even after 6 or 24 h (data not shown). This is strengthening the assumption that COXII - or rather the domain of COXII that is detected - must be processed by a protease first in order to

be detected in the extract, otherwise it will stay tethered to the membrane. The histone H3 signal exhibits a size shift towards a smaller molecular weight. In combination with the result of the DNA laddering analysis, it can be assumed that this size shift is caused by the degradation of the associated DNA on the Histones. DNA fragments on the histone will add molecular weight to it and can also interfere with SDS molecules during the SDS-PAGE run as both DNA and SDS are negatively charged and will repel each other, causing the SDS molecules to "slip off" the protein. An alternative explanation for the size shift of the Histone over time could be degradation of the protein itself. Incubation with protease inhibitor has shown to inhibit size shift (data not shown), however, the protease inhibitor also contains EDTA, an agent that sequesters calcium and magnesium ions which are important co-factors for the function of nucleases. We therefore also observed an inhibitory effect of the protease inhibitor on DNA laddering (data not shown).

Overall we can hypothesize that PEF treatment under these parameters induces irreversible membrane permeabilization and in consequence programmed cell death in *C. vulgaris*. The protein release is likely facilitated by autolytic processes associated with programmed cell death, which have been proposed and described for other microorganisms such as yeast already [29,30,33] This process can be influenced by environmental factors such as incubation temperature and pH. A further look into these circumstances could greatly contribute to the design of a processing cascade to efficiently fractionate microalgae biomass into its valuable compounds while saving energy since it has also been described that autolytic processes can be induced at lower energies than used in this study and have been applied to extract pigments from *Chlorella vulgaris* [34,35].

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Declaration of contributions

Conception and design of the experiments: DS, CG, WF, GM, PN. Experiments: DS & DK. Microalgae cultivation: DS & DK. Draft of this article: DS. Revision of this article: DK, CG, WF, GM, PN.

DS takes responsibility for the integrity of this work.

Conflict of interest

The authors declare no conflict of interest.

Statement of informed consent

No conflicts, informed consent, human or animal rights applicable.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the corresponding author is the sole contact for the editorial process.

He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

Signed by all authors as follows: DS, DK, WF, GM, PN, CG.

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