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Effect of precipitation, lyophilization, and organic solvent extraction on preparation of protein-rich powders from the microalgae *Chlorella protothecoides*

L. Grossmann[a], S. Ebert[a], J. Hinrichs[b], J. Weiss[a,*]

[a] University of Hohenheim, Department of Food Physics and Meat Science, Institute of Food Science and Biotechnology, Garbenstrasse 21/25, 70599 Stuttgart, Germany
[b] University of Hohenheim, Department of Soft Matter Science and Dairy Technology, Institute of Food Science and Biotechnology, Garbenstrasse 21, 70599 Stuttgart, Germany

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ABSTRACT

The aim of this study was to investigate the production of protein-rich powders from liberated proteins of the heterotrophically cultivated microalgae *Chlorella protothecoides* by means of precipitation, lyophilization, and solvent extraction, and assess the characteristics of the obtained materials. First, microalgae dispersions (10 g/100 g) were passed up to 12 times through a high-pressure homogenizer at different pressure levels. Second, the cells were centrifuged (20,000*g*, 30 min, 25 °C) to obtain soluble- and insoluble-protein fractions. Lastly, three approaches were used to obtain protein-rich powders from both fractions: (a) solvent precipitation, (b) lyophilization, and (c) solvent extraction. Results showed that cells of *Chlorella protothecoides* could be disrupted by high pressure homogenization, e.g. 6 passes at 150 MPa led to 99.9% cells being disrupted. For protein precipitation, solvent polarity affected precipitation efficiency in terms of protein and pigment content of the obtained powders. Precipitation with an ethanol:acetone (1:1 *v*/v) mixture resulted in overall high protein contents in the obtained powders of 46.3 ± 0.1 g/100 g from soluble fractions and 67.2 ± 1.0 g/100 g from insoluble fractions. Solvent extraction of lyophilized powders had only slight effects on protein and pigment content but lyophilized powders itself had a protein content of 39.1 ± 0.5 g/100 g (soluble powder) and 58.5 ± 0.5 g/100 g (insoluble powder). The three applied methods yielded in powders with different characteristics, which might allow use in different food formulations.

1. Introduction

Microalgae are a highly diverse polyphyletic group of unicellular eukaryotic and prokaryotic photosynthetic organisms. They are considered to be one of the first existing organisms on this planet, dating back around 3465 million years [1]. The selective cultivation of microalgae has gained attention because of their high productivity, which is related to their high growth rate and photosynthetic efficiency [2,3]. Additionally, microalgae accumulate high amounts of valuable macro- and micronutrients, such as proteins. Depending on the microalga, feeding, and cultivation conditions, protein contents of up to 71% (in dry matter) have been reported [4]. Considering an estimated growth of the world population to around 10 billion by 2050, which will greatly increase protein demand, microalgae might become an additional protein source for food purposes in the future [5].

The microalgae genus of *Chlorella* is one of the most often cultivated and intensely studied microalgae. *Chlorella* is a green alga and can be cultivated phototrophically, mixotrophically, and heterotrophically. Recent studies showed that the *Chlorella* species *Chlorella protothecoides* accumulates high protein contents (40–70%) during axenic fermentation and safety evaluations indicated applicability for food formulations [6,7]. The advantage of this microalga compared to other *Chlorella* species is the absence of chlorophyll after heterotrophic cultivation, which is difficult to remove from the cells, and its lack of *N*-acetylglucosamine in the rigid cell wall, which enhances the rigidity of the cell wall [8]. To fully exploit the functionality of these cell proteins in foods, they have to be liberated from the cells and separated from other cell ingredients. Here, one major challenge is the high integrity of the cell wall, which makes cell lysis quite difficult. This integrity is based on the cell wall thickness and the composition of the cell wall itself. The overall cell wall thickness has been reported to range from 17 to 22 nm for different *Chlorella* species, which have a cell size of 2–10 µm [7,9]. In case of *Chlorella protothecoides*, it consists of one rigid part, which mainly contains glucose-mannose polymers [8]. This rigid cell wall is

* Corresponding author.
E-mail address: j.weiss@uni-hohenheim.de (J. Weiss).

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Table 1
Main studies that investigated the extraction and formation of protein-rich powders from different microalgae.

Microalgae	Cell disruption prior to treatment	Protein separation	Challenge	
Dunaliella tertiolecta Butcher, Amphidinium carterae Hulburt, Skeletonema costatum, Isochrysis galbana Parke, Hillea sp.	–	Trichloroacetic acid precipitation	Low sample amount (50 mg), harsh chemicals (trichloroacetic acid, β-mercaptoethanol), low protein contents in the precipitate (≤ 25%)	[11]
Tetraselmis sp.	Bead mill	Expanded bed adsorption, isoelectric point precipitation	Energy consumption due to dialysis and expanded bed adsorption, low protein solubility at low pH-values (circa 20% at pH 4)	[14]
Nannochloropsis spp.	Ultrasonication	Isoelectric point precipitation	Possible protein denaturation during hot pre-treatment (80 °C)	[12]
Chlorella vulgaris	High pressure homogenization	Isoelectric point precipitation, ultrafiltration	No cooling during cell disruption, decreasing precipitation yield with decreasing pH (from 76 ± 4% at pH 12 to 57 ± 4% at pH 7), resolubilization only in alkaline conditions	[16]
Scenedesmus incrassatulus	–	Ethanol extraction (50 °C), enzymatic proteolysis of extracted biomass following protein fractionation by centrifugation and subsequent lyophilization	Protein degradation at elevated temperatures (50–70 °C), change in native structure due to proteolysis	[40]
Scenedesmus sp.	–	Flash hydrolysis with subcritical water and freeze drying	Protein degradation at high temperatures (205–325 °C)	[41]
Spirulina platensis	–	Isoelectric point precipitation	No yield stated	[42]

embedded in a polymeric matrix that consists of uronic acids and neutral sugars [10].

A substantial amount of research has been carried out on microalgae cell disruption for the purpose of lipid extraction. Cell disruption with the aim of protein liberation has been carried out to a lesser degree for a few different microalgae [11–16]. In the case of *Chlorella*, several studies concluded that high pressure homogenization is the most effective method to liberate proteins from microalgae cells [17–19]. To the best of our knowledge, cell disruption studies using high pressure homogenization with the aim of protein liberation from *Chlorella protothecoides* have not yet been done.

The liberated proteins might be precipitated from aqueous solutions using different principles such as salting-out, isoelectric point precipitation, and solvent precipitation. A short summary of the main studies that dealt with protein extraction with the aim of generating a protein-rich powder is shown in Table 1. The key challenge described was to preserve the native state of the protein as much as possible while maintaining scalability of the separation process. Other authors have used predominantly isoelectric point precipitation to separate proteins from aqueous microalgae solutions [12,14,16,19]. However, these authors did not separate the proteins into a water-soluble and water-insoluble fraction, which might lead to a different effect in each fraction during precipitation. In contrast, solvents such as ethanol, acetone, chloroform, and hexane were mainly used to extract lipids and pigments from microalgae, whereas in other research areas solvents are frequently used to precipitate proteins from aqueous solutions. Especially the high pigment content is a major challenge for the application of microalgae products, because microalgae can't be easily incorporated into common products due to the strong alteration in color. For these reasons, proteins were initially liberated and fractionated from *Chlorella protothecoides* cells into a soluble- and insoluble-protein fraction by homogenization and centrifugation. Subsequently, protein precipitation at the isoelectric point and by the addition of solvents to both fractions in an untreated and in a lyophilized state was examined. The overall aim of the study was to obtain protein-rich powders from both fractions that can be used in further studies to evaluate the technofunctionality of the microalgae proteins in food formulations in detail.

2. Materials and methods

2.1. Materials

Heterotrophically and specifically cultivated for protein accumulation *Chlorella protothecoides* cells (Algility™ HP) were purchased from Roquette Frères and the proximate composition is outlined in Table 2 (Lestrem, France). 2-Mercaptoethanol (≥ 99%, p.a., #4227), acetone (≥ 99.5%, #5025), anthrone (p.a., ACS, #2673), D-Glucose (p.a., ACS, anhydrous, #X997), ethylenediaminetetraacetic acid (≥ 99%, p.a., ACS, #8040), n-hexane (≥ 99%, #T861), hydrochloric acid (≥ 32%, p.a., ISO, #P074), petroleum ether 40–60 °C (p.a., ACS, ISO, #T173), sodium hydroxide (≥ 99%, p.a., ISO, #6771), sulfuric acid (96%, p.a., ISO, #4623) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Ethanol (purity 100%, containing 1% butanone) was purchased from BrüggemannAlcohol Heilbronn GmbH (Heilbronn, Germany). Glycine (≥ 99%, #G8898), HYDRANAL®-Titrant 2 (#34811), and HYDRANAL®-Solvent (#34800) were purchased from Sigma-Aldrich (Steinheim, Germany). Pierce™ BCA Protein Assay Kit including albumin standard ampules (#23227) was obtained from Fisher Scientific GmbH (Schwerte, Germany). Coomassie Brilliant Blue R-250 (#1610436) was bought from Bio-Rad Laboratories GmbH (Munich, Germany).

2.2. Methods

The protocol of the complete experimental setup is outlined in Fig. 1.