Evaluation of pretreatment methods for primary recovery and capture of an antibody fragment (α CD22scFv) from *Chlamydomonas reinhardtii* lysates

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Abstract: Optimum conditions for extraction of an acidic (pI 5.1) single-chain antibody fragment (α CD22scFv) produced in the chloroplast of *C. reinhardtii* were determined as function of pH, NaCl, and Tween 20. The maximum extraction yield of α CD22scFv was achieved at pH 8 with 400 mM salt and 0.5% Tween. The effectiveness of mechanistically different precipitation methods (polymer, isoelectric, and salt) for reduction of DNA, chlorophyll, and host cell protein in cell lysates and clarified cell lysates was compared. Acid and ammonium sulfate precipitation significantly reduced host cell protein and chlorophyll in cell lysates and clarified cell lysates with a minimal loss of α CD22scFv. Precipitation of cell lysates and clarified cell lysates with chitosan (cationic polymer) was the best overall pretreatment method and was accompanied by 10-15% lower α CD22scFv recovery yield compared to the other two methods. To check compatibility of pretreated extracts with non-affinity adsorption chromatography, batch adsorption experiments of α CD22scFv from pretreated extracts were performed. Ammonium sulfate (1M) pretreated extracts were not suitable for direct capture of α CD22scFv by Phenyl Sepharose resin because the target protein could not be adsorbed completely even in the presence of 1.5M ammonium sulfate. α CD22scFv completely adsorbed onto Capto Q resin at pH 8 from acid- and chitosan-pretreated extract after reducing the extracts ionic strength to 4 mS. Capture

chromatography of α CD22scFv from chitosan- and acid-pretreated extracts resulted in 20- and 13- purification fold, respectively.

Keywords: Single-chain antibody fragment, *Chlamydomonas reinhardtii*, clarification, precipitation, extract impurities, capture chromatography

1. Introduction

Advances in microalgae biotechnology in the past 15 years are built on early development successes of transgenic plants as bioreactors for recombinant protein production [1]. The ability to transform several microalgal species (*Chlamydomonas reinhardtii*, *Dunaliella salina*, and diatom *Phaeodactylum tricornutum*) for heterologous genes expression opened the door for exploring the development of microalgae as a platform for biopharmaceuticals and industrial protein production. The interest in eukaryotic microalgae as an alternative platform for recombinant proteins stems from several relative advantages of microalgae compared to bacteria and plants as reviewed by Specht et al. [2]. *Chlamydomonas reinhardtii* chloroplast is currently the preferred target location for production of recombinant proteins with no glycosylation requirement for functioning, and more than twenty different proteins have been expressed in *C. reinhardtii* chloroplast alone [3-5]. Extraction of intracellular proteins from microalgae is similar to plant tissue with exception of phenolics algae extracts contain cell debris, host cell protein, nucleic acid, and chlorophyll pigments. To maximize the recombinant protein recovery from leafy plant tissues, optimization of biomass-to-buffer ratio, pH, and ionic have been successfully employed [1,6-10].

Clarification of cell lysates can be challenging and the removal cell debris and suspended particles by centrifugation or microfiltration is often not efficient resulting in turbid supernatants that require several depth filtration steps before the first chromatography column[11]. To increase clarification efficiencies and process robustness, green tissue extracts have been pre-processed by using ammonium sulfate precipitation [12], isoelectric precipitation [9,13] and polymer-mediated flocculation or precipitation [14,15]. For example, low saturation ammonium sulfate solutions (25–30%) have been

applied to reduce concentrations of native plant proteins, protein aggregates, and cell debris [12,16]. Acidification of leafy extracts and cell homogenates to pH <5.0 led to precipitation of the most abundant plant protein (rubisco), cell debris, as well as chlorophyll pigments attached to the protein and debris [16,9]. Cationic polymers, such as polyethylenimine (PEI) and chitosan, have been used to flocculate whole cells and cell debris [17,14,18], remove nucleic acids [15], or to precipitate recombinant β -glucuronidase (pI 5.5) from low ionic strength tobacco extracts [14]. In this study, cationic chitosan polymer was chosen for this investigation because hydrophilic chitosan polymer backbone exhibits a greater ionic-strength tolerance than PEI.

Although the precipitation and flocculation methods listed above have been applied before as process agents for protein precipitation, DNA removal or lysate clarification, their *a priori* effect on algal DNA, chlorophyll, and host cell protein removal and on product recovery yield is not clear. The two main challenges with regard to precipitation efficacy of acidic recombinant protein products in plant and algal extracts are i) acidic properties and abundance of native proteins (rubisco) and ii) high salt concentration required for the release of intracellular recombinant proteins [1]. Conditions that favor formation of native (rubisco) proteins precipitation may also be favorable for co-precipitation of acidic recombinant proteins, whereas high-ionic strength of the extracts interferes with protein-polymer complex formation process that relies on charge interactions. The end result of either situation is reduced precipitation efficacy.

After conditioning of the extracts, purification usually starts with a capture chromatography step to concentrate the target recombinant protein and remove critical impurities. The purification strategy employed for algae or plant based systems is similar to the other protein production systems and is mainly dependent on protein properties and host cell impurities [1]. Various chromatography resins are screened for functionality and optimized uniquely for optimum capture of a specific target recombinant protein. There have been very few studies reporting purification strategies for algae-derived recombinant proteins. Some of the plant-derived recombinant proteins have been captured by anion-

exchange adsorption [19], cation exchange adsorption [20], affinity [21] and hydrophobic interaction chromatography [22].

In this study, we investigated the potential of three mechanistically different precipitation methods (chitosan, ammonium sulfate, and acid precipitation) for pretreatment and conditioning of algal cell lysates and clarified cell lysates followed by the evaluation of its compatibility with the capture chromatography step. The objectives of this work were: 1) to determine the optimal extraction conditions of an acidic single-chain antibody fragment (α CD22scFv) produced in the chloroplast of *C. reinhardtii*, 2) to compare suitability and efficiency of chitosan, acid, and ammonium sulfate precipitation for pretreatment of algal lysates, and 3) to determine the compatibility of extract pretreatment to the subsequent recombinant protein capture step.

2. Experimental Section

2.1. Gene constructs for α CD22scFv

In the construct, the endogenous *psbA* locus was replaced by α CD22scFv via direct homologous recombination. Thus, transgene expression in these strains is regulated by the *psbA* promoter and the 5' and 3' untranslated regions (UTRs) and, therefore, is light inducible. A kanamycin resistance cassette was incorporated for selection. The variable domains of a human antibody against the B-cell surface antigen CD22 were separated by a linker consisting of four glycines and a serine repeated four times (4×G4S) to create an scFv [4]. The gene cassettes (α CD22scFv) was ligated with a sequence coding for a 1× Flag peptide (DYKDDDDKS) and separated by a sequence that encodes a tobacco etch virus (TEV) protease cleavage site (ENLYFQG) [4].

2.2. Cultivation of recombinant α CD22scFv *C. reinhardtii* strains

Algal biomass from a single tris-acetate-phosphate agar plate containing 150 μ g/mL kanamycin was transferred to 100 mL of TAP (tris-acetate-phosphate) media without kanamycin and grown for 3 days.

Subsequent volumetric culture scale up was performed using 10 % inoculum in the exponential phase (100 mL) in 1-L of fresh TAP media containing 25 μ g/mL kanamycin. One liter cultures were grown heterotrophically (in the dark) for 5 days reaching ~ 4 to 5×10^5 cells/mL. At the end of the fifth day, the biomass from 1-L cultures was resuspended in 1-L of fresh TAP media containing 25 μ g/mL kanamycin and grown for 1 day reaching about 10^6 cells/mL. The cultures were then exposed to light at 300 μ mol m⁻²s⁻¹ for 24 hours to induce recombinant protein synthesis. Cell growth was monitored daily by counting cells using a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA) and measuring optical density at 750 nm using a Beckman Coulter DU640 spectrophotometer (Brea, CA).

2.3. Protein extraction

C. reinhardtii cultures producing recombinant proteins were grown in liquid media until they reached the desired cell concentration of $\sim 10^6$ cells/mL. At the end of the light exposure period, cells were harvested by centrifugation at 10,000 $\times g$ for 15 min at 4 °C. Pelleted algal biomass was washed with fresh TAP media, weighed, and then resuspended in 50 mM Tris-HCl, 400 mM NaCl, and 0.5% Tween 20, pH 8.0 lysis buffer at a 1:5 biomass-to-buffer ratio. The buffer contained one cOmplete™ ULTRAprotease inhibitor cocktail tablet (Roche-Mannheim, Germany) dissolved in 200 mL of the buffer. Algal cells were lysed by sonication for 8 min with 30 s on/off intervals at 4°C using sonicator (Sonifier 250, Branson, USA) at 30% output control and 30% duty cycle with a micro probe (1/8" microtip A3-561 Branson, USA). Cell lysates were centrifuged (10,000 $\times g$ for 10 min) to produce clarified cell lysates.

For optimization of extraction conditions of α CD22scFv, buffer composition (pH, NaCl, Tween 20) was varied depended on the extraction objective. Two levels each for pH (4.5 and 8), NaCl (0 mM, 400 mM) and Tween 20 (0% and 0.5%) were tested. A 2³ full factorial design was performed to determine the effect of salt NaCl, detergent Tween 20, and pH on culture concentration of recombinant protein α CD22scFv. All buffers contained a complete protease inhibitor cocktail (Roche-Mannheim,

Germany) dissolved in 200 mL of the buffer. Cell lysates were centrifuged (10,000 x g for 10 min) and filtered with PES 0.45 µm syringe filters to produce clarified extracts.

2.4. Pretreatment of clarified cell lysates and cell lysates

The cell lysate or clarified cell lysate obtained after sonication in optimum extraction buffer was subjected to three different pretreatments, i.e. ammonium sulfate precipitation, acid precipitation and polymer flocculation/precipitation followed by centrifugation and depth filtration (Figure 1). Each pretreatment was compared to control (no-pretreatment) from the same algae batch and was done in triplicates.

Ammonium sulfate (Sigma Aldrich Company, USA) precipitation was performed by adding the calculated amount of 3.6 M ammonium sulfate stock solution to the *C. reinhardtii* cell lysates or clarified cell lysates to achieve the desired molar concentration (0.5M, 1.0M, 1.5M, 2 M). The lysates or clarified cell lysates were then mixed (end over end) for 30 minutes at room temperature followed by centrifugation at 10,000 x g for 10 min. The supernatants were filtered with PES 0.45 µm syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and αCD22scFv by methods discussed below.

Figure 1. Primary recovery steps of αCD22scFv from *C. reinhardtii* lysate

Acid precipitation was performed by adding 0.5 N HCl (dropwise with gentle shaking) to *C. reinhardtii* cell lysates or clarified cell lysates until the pH dropped to 4.5. Agitation was provided by end-over-end gentle mixing for 15 min, followed by centrifugation at 10,000 x g for 10 min. The acid supernatants were adjusted to pH 8 with 1 N NaOH. The supernatants were filtered with PES 0.45 μ m syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and α CD22scFv.

Polymer pretreatment was carried out on cell lysate or clarified cell lysate with 10 mg/mL stock solution of chitosan in 1% acetic acid solution (Sigma Aldrich Company, USA). The stock solution of chitosan was prepared by mixing chitosan in a 1% acetic acid solution for 24 h at 60 x.g and room temperature. For both, cell lysate and cell free extract, thirty milligrams of chitosan (3 ml stock) was added per gram of algae biomass followed by vortexing for 30 sec. The lysate pH was then adjusted to 5.0 with 0.1 N HCl and mixed end-over-end for 30 min at room temperature (22°C). The cell lysate pH was then adjusted back to pH 8.0 with 1 M NaOH to precipitate unreacted chitosan and then clarified by centrifugation at 6000 x g for 10 min. The supernatant was filtered with PES 0.45 μ m syringe filter and analyzed for host cell protein (HCP), DNA, chlorophyll, turbidity, and α CD22scFv by methods discussed below. The same sequence of pH adjustments followed by centrifugation and filtration was used with clarified cell lysates.

2.5. Determination of α CD22scFv in cell-free lysates and pretreated extracts by FLAG affinity adsorption

The FLAG affinity purification described previously was used to estimate the recombinant protein extraction and recovery yield [23]. Cell-free and pretreated extracts described above were mixed with anti-FLAG affinity resin (Sigma Aldrich A4596) equilibrated in the extraction buffer (50 mM Tris-HCl, 400 mM NaCl, and 0.5% Tween 20, pH 8.0.). Binding of the recombinant protein to the affinity resin was performed for 2 h at 4 °C by continuous end-over-end mixing in a Glass Col rotor at ~ 33 x g (40 % speed control). Affinity resin was washed with 10 column volumes (CV) of lysis buffer followed by 3 column volume of lysis buffer without Tween. The washed FLAG resin was transferred

into Bio Spin disposable chromatography columns (Bio Rad) for protein elution at room temperature. α CD22scFv was eluted at pH 3.5 using 5 CV of 100 mM glycine buffer, pH 3.5 that contained 400 mM NaCl. Eluted protein fractions were immediately adjusted to pH 8.0 with 1M Tris-HCl. Clarified cell lysates, pretreated extracts, and adsorption supernatants were regularly analyzed by western blotting to assure complete extraction and adsorption, respectively.

2.6. Protein analysis

Filtered algal crude extract and purified samples were analyzed by SDS-PAGE, Western blot, and total eluted protein determined by Bradford assay [24]. Host cell protein/total soluble protein from crude extract and purified samples were quantified using the microplate protocol (working range from 1 to 25 μ g/mL and 25 to 1500 μ g/mL) Coomassie plus [24] assay kit (Thermo Scientific). Absorption at 595 nm was measured using the VERSA max microplate reader (Molecular Devices).

NuPAGE Novex Bis-Tris pre-cast gradient gels (4 - 12 %) from InvitrogenTM (1.5 mm x 10 wells), (Cat no. NP0335BOX) were used for SDS-PAGE electrophoresis. Reducing buffer was prepared using NuPAGE LDS sample buffer (4X) containing NuPAGE sample reducing agent. Reduced samples were prepared using a 1:4 ratio reducing-buffer: sample and heated at 70 °C for 10 min. NuPAGE MES SDS Running Buffer (20X) stock solution was used to prepare 1X running buffer in RO water. NuPAGE antioxidant was added to ensure reduced samples during electrophoresis. Gels were run for 35 min at constant voltage (200 V). For SDS analysis, the gels were stained in CoomassieTM G-250 stain (ThermoFisher Scientific) for 3 hrs followed by destaining in RO Water. For Western blot analysis, the gel was transferred to nitrocellulose membranes using iBlot® 7-Minute Blotting System (Life Technologies Corporation).

After protein transfer to a nitrocellulose membrane, the membrane was blocked with 2.5 % non-fat milk in TBS containing 0.05 % Tween 20 at pH 7.5 buffer for 1 h to prevent nonspecific binding of the detection antibodies. FLAG-tagged α CD22scFv was detected by using anti-FLAG M2-AP (alkaline

phosphatase conjugated) antibody from Sigma Aldrich (A9469) at a concentration of 1:1,000. After incubation with the antibody for 1 h, the membrane was washed with TBS containing 0.05 % Tween 20 at pH 7.5, buffer and blots were visualized with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP) (Sigma FAST B5655) dissolved in 10 mL of filtered RO water.

2.7. Batch adsorption of α CD22scFv from pretreated extracts

To evaluate the effect of different pretreatments on non-affinity capture of α CD22scFv from clarified extracts, a set of batch adsorption screening experiments were carried out. Chitosan - and acid-pretreated extracts were contacted with an anion exchange resin (Capto Q) and ammonium sulfate precipitated ones with hydrophobic interaction resin (Phenyl-Sepharose).

Acid, chitosan, and ammonium sulfate precipitation was performed on clarified cell lysates as described previously. Acid and chitosan pretreated samples were dialyzed against 50 mM Tris-Base pH 8.0 to reduce the conductivity of the lysate to below 4 mS. The extract was added to SnakeSkin[®] Dialysis Tubing 3,500 MWCO (Thermo Scientific, Meridian Rd., Rockford, IL, USA) and left in the exchange buffer for 24 hrs in a 4°C cold room. After dialysis, the extracts were incubated with fresh Capto[™] Q (Sigma Aldrich, St. Louis, MO, USA) resin for 30 min, which was followed by centrifugation for 5 minutes at 3,000 x g to settle the resin. The resin was loaded onto a BioSpin disposable chromatography column (Bio Rad, Hercules, CA, USA), washed with 10 CVs of washing buffer (50mM Tris-Base, pH 8.0), and eluted step-wise with washing buffer plus increasing amounts of salt (0.2, 0.4, 0.6, 0.8, 1.0M NaCl). Two CVs of elution buffer was used before moving to the next elution buffer of additional NaCl. Supernatants at the end of the batch adsorption period, washes, and elutions were analyzed by Western blotting to evaluate adsorption and specific binding to the resin.

Ammonium sulfate pretreated sample were incubated with Phenyl Sepharose[™] 6 Fast Flow (high sub) (Sigma Aldrich, St. Louis, MO, USA) resin on an end-over-end rotor at room temperature for 30 min.

After incubation, the resin was centrifuged for 5 minutes at 3,000 x g to settle the resin and then transferred to a Bio Spin disposable chromatography column (Bio Rad, Hercules, CA, USA). The resin was then washed with 10 CVs of washing buffer (50mM Tris-Base, 1 M ammonium sulfate, pH 8.0) and eluted using washing buffer with decreasing amount of ammonium sulfate from 0.8 to 0 M in increments of 0.2 M ammonium sulfate.). Two CVs of elution buffer was used before moving to the next elution buffer of less ammonium sulfate. Supernatants at the end of the batch adsorption period, washes, and elutions were analyzed by Western blotting to assess adsorption and specific binding to the resin.

2.8. Anion exchange chromatography of pretreated extracts

Since acid precipitation-Capto Q and chitosan precipitation-Capto Q were compatible pretreatment-capture step combinations, these processes were scaled up using AKTA™ Purifier (GE Healthcare Life Sciences, Piscataway, NJ, USA). Acid precipitation and chitosan precipitation was carried out on cell-free lysate as outlined above. Acid and chitosan pretreated samples were dialyzed against 1 L of 50 mM Tris Base at pH 8 to bring the conductivity of the samples below 4 mS. The lysate was added to SnakeSkin® Dialysis Tubing 3,500 MWCO (Thermo Scientific, Meridian Rd., Rockford, IL, USA) and left in the exchange buffer for 24 hrs in a 4°C cold room. Dialyzed samples were loaded at 30 cm/h onto pre-packed HiScreen Capto-Q column (GE Healthcare Life Sciences, Piscataway, NJ, USA) The column was washed with 10 CVs of washing buffer (50 mM Tris Base, pH.8, 1.8 mS). Gradient elution was performed for 20 CVs with washing buffer and increasing concentration of salt (0 to 1 M NaCl, 1.8 to 77 mS). Both washing and elution were performed at 30 cm/h. The amount of α CD22scFv in cell-free lysate, pretreated samples, and pooled elution fractions containing α CD22scFv, was estimated using anti-FLAG affinity resin.

2.9. DNA determination

The DNA concentration in the algae extract was determined using a Quant-iT™ PicoGreen® dsDNA Reagent Kit from Molecular Probes (Eugene, OR). The assay was performed using a fluorescence plate reader, Spectra Max Gemini XS, from Molecular Devices following the procedure recommended by the manufacturer. The samples were excited at 480 nm, and the fluorescent emission intensity at 520 nm was measured. The assay limit is 250 pg/mL. Standard curve (Range 1-1000 ng/ml) was made using lambda DNA standard present in the kit.

2.10. Chlorophyll determination

Chlorophyll (a and b) was measured by measuring the optical density at 652 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer. Blanks were prepared with lysis buffer containing ammonium sulfate or chitosan for respective pretreatments.

2.11. Turbidity measurement

It was measured by measuring the optical density at 750 nm wavelength using a Beckman Coulter (Brea, CA) DU640 spectrophotometer. Blanks for pretreated samples contained ammonium sulfate or chitosan, respectively.

2.11. Statistical analysis

Design Expert software was used for the experimental design and analysis. The statistical significance of the models was evaluated by the analysis of variance (ANOVA). Significantly different means ($p < 0.05$) were separated by the Tukey's test.

3. Results

3.1. Extraction optimization of α CD22scFv

Previously, we have established that the optimization of algal growth and induced-expression conditions could significantly enhance recombinant protein concentration in algal biomass [23].

Another way to further enrich algae extracts in recombinant protein concentration is to examine the effect of key extraction variables on recombinant protein yield and the concentration of host cell impurities. A biomass-to-buffer ratio of 1:5 (instead of 1:10) was used to reduce the process volume without compromising the amount of extracted recombinant protein. Table 1 compares the effect of different extraction conditions on the concentration of chlorophyll, host cell protein, DNA, and α CD22scFv in clarified extracts. Each factor was studied on two levels: pH (4.5 and 8), NaCl (100mM and 400mM) and Tween (0% and 0.5%). The specific levels of all three factors were chosen based on previous extraction data of recombinant proteins from transgenic plant tissues and algae [9,13,4,25]. The concentrations of measured impurities and recombinant protein were normalized against those obtained at pH 8.0, 0.4 M NaCl, and 0.5% Tween. At pH 8, both Tween 20 and 400 mM salt were required for maximum extraction of α CD22scFv. Omitting Tween from the extraction buffer reduced the concentration of both α CD22scFv and HCP by 29% and 22%, respectively. Chlorophyll concentration at pH 8 was affected (25% reduction) only when Tween was removed from the buffer and salt concentration was reduced to 100 mM. The latter two concentrations further reduced the extracted HCP and α CD22scFv by about 20%. DNA concentration at pH 8 was not affected by any of tested variables. At pH 4.5, Tween alone had a pronounced effect on extracted chlorophyll at both salt concentrations, reduction by 45% and 60%. HCP was reduced from 62% to 22% in the absence of Tween if pH 4.5 buffer contained 400 mM salt. DNA concentration was significantly reduced (by 40 %) only at pH 4.5 with no Tween and 100 mM salt. α CD22scFv concentration remained at about 70% for all pH 4.5 tested conditions. It was thus observed that buffer of high pH with high salt and Tween 20 maximizes host cell protein extraction, whereas pH 4.5 and low or no salt in the extraction buffer minimizes the amount of extracted host cell protein. In conclusion, a combination of low pH buffer, low ionic strength (low salt concentration) and absence of Tween in the lysis buffer could significantly reduce chlorophyll (70%) and total soluble protein (55%) concentrations, but under those conditions a loss of 30% α CD22scFv was also observed.

Chloroplast expressed proteins may require detergents Tween to reduce hydrophobic interaction with thylakoid membranes similarly to recombinant proteins expressed in tobacco chloroplast [26,27], but this effect has been observed at pH 8 and not at pH 4.5. Our data suggest that the recovery of chloroplast-expressed protein is somewhat related to the solubilization of host-cell protein and probably to reduction of hydrophobic protein-protein and protein-membrane interactions. The maximum recovery yield of α CD22scFv was achieved at pH 8 with 400 mM salt and 0.5% Tween 20, the conditions that also maximize HCP, DNA, and chlorophyll extractability. Adjusting the pH of the algae lysate below pH 5.0 reduces solubility of the most abundant protein (rubisco), which has a pI of 5.1 and indirectly chlorophyll pigments nonspecifically bound to host cell protein. The addition of Tween 20 at pH 4.5 increased chlorophyll solubility and host cell protein, but did not result in measureable change in α CD22scFv concentration. The extraction data suggest that pH 4.5 buffer with 100 mM salt and no Tween 20 would be optimal with respect to extract impurities if 70% α CD22scFv extraction yield were acceptable.

Table 1: Effect of extraction conditions (pH, NaCl, Tween) on normalized concentrations of chlorophyll, HCP, α CD22scFv and DNA. Values given are averages from 3 replicates $\pm$ standard deviations. ^{a,b,c} For each observation, means within a column which are not followed by a common superscript letter are significantly different ($p < 0.05$).

Extraction conditions			Criteria studied			
pH	NaCl (mM)	Tween %	Chlorophyll (%)	HCP (%)	α CD22scFv (%)	DNA (%)
8	400	0.5	100 ^a $\pm$ 6.7	100 ^a $\pm$ 13	100 ^a $\pm$ 14	100 ^a $\pm$ 10
8	400	0	98.3 ^a $\pm$ 0.5	78.3 ^b $\pm$ 16	70.9 ^b $\pm$ 1.9	115 ^a $\pm$ 2.2
8	100	0.5	95.8 ^a $\pm$ 3.9	103. ^a $\pm$ 4.0	52.4 ^b $\pm$ 20	106 ^a $\pm$ 8.7
8	100	0	75.0 ^b $\pm$ 3.5	56.0 ^b $\pm$ 16	62.5 ^b $\pm$ 16	135 ^a $\pm$ 18
4.5	400	0.5	73.5 ^b $\pm$ 4.9	61.5 ^b $\pm$ 9.9	62.0 ^b $\pm$ 24	128 ^a $\pm$ 25
4.5	400	0	28.5 ^c $\pm$ 2.0	21.6 ^c $\pm$ 8.3	74.0 ^b $\pm$ 4.2	92.2 ^a $\pm$ 8.3
4.5	100	0.5	77.8 ^b $\pm$ 4.0	50 ^b $\pm$ 15.3	70 ^b $\pm$ 21	98.2 ^a $\pm$ 7.2

4.5	100	0	17.9 ^d ± 0.9	45.7 ^b ± 0.9	71.1 ^b ± 5.5	61.9 ^b ± 4.2
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307 3.2. Evaluation of pretreatment methods in primary protein recovery

308 Extraction conditions that maximize product recovery are often also optimal for the release of a variety
 309 of host cell proteins and other water soluble cell components. Since these components could affect the
 310 final product quality and reduce the lifetime of capture chromatography resin, ammonium sulfate, acid,
 311 and chitosan precipitation were compared and evaluated as potential pretreatment methods for
 312 clarification and impurities reduction of *C. reinhardtii* cell lysates and clarified cell lysates (Figure 1).
 313 These three pretreatment methods were chosen because of their common underlying action mechanism
 314 (precipitation) and subsequent processing steps - centrifugation and depth filtration.

315 The initial screening of the effect of ammonium sulfate concentration on host cell protein and
 316 chlorophyll reduction was done using clarified cell lysates (Figure 2). Data in Figure 2 show that
 317 increasing the concentration of ammonium sulfate from 0.5 to 2.0 M resulted in a linear reduction of
 318 host cell protein. Chlorophyll reduction was proportional in the 0.5-1.5 M range and further increase
 319 on ammonium concentration did not result in additional reduction. The impact of ammonium sulfate
 320 pretreatment on the amount of residual α CD22scFv in the supernatant was measured at each
 321 ammonium sulfate concentration and expressed as percent recovery (numbers in parenthesis in Figure
 322 2). Because the amount target recombinant protein was significantly reduced (48%) when using 1.5 M
 323 ammonium sulfate, a pretreatment by 1M ammonium sulfate precipitation was chosen as a reasonable
 324 compromise between host cell protein and chlorophyll removal and product loss.

Figure 2. Effect of ammonium sulfate concentration on residual host cell protein (HCP) and chlorophyll in clarified cell lysates after precipitation. Values plotted are averages from 3 replicates; bars represent standard deviation. Numbers in parenthesis represent α CD22scFv yield in respective supernatants.

The effect of the pretreatment methods on algae lysates and cell free extracts is summarized in Table 2. DNA, chlorophyll pigments, host cell proteins, and recombinant protein α CD22scFv after each pretreatment were determined and normalized against the control. Control experiments (no pretreatment) were performed from the same algae batch to minimize the effect of batch-to-batch variability. The host cell protein of the extract treated by ammonium sulfate precipitation was reduced by 44% in cell-free lysate and 54 % in cell lysate. Host cell protein of extracts treated by acid precipitation was reduced by 69% in cell-free lysate and by 64% in cell lysate. In the case of chitosan precipitation, host cell protein was reduced by 82% in both cell lysate and clarified cell lysate. Chlorophyll content showed a wide range among the pretreatments tested. Chlorophyll was reduced by 40% in cell-free lysate and by 70% in cell lysate with ammonium sulfate precipitation, and by 72% in cell- free lysate and by 45% in cell lysate with acid precipitation. Chitosan precipitation significantly reduced (99%) chlorophyll in clarified cell lysate, but it was less efficient (58%) in cell lysate.. The positively charged chitosan was very efficient in reducing DNA content by 91% in cell-free lysate and

by 86% in cell lysate. On the other hand, and not entirely unexpected, acid precipitation and ammonium sulfate precipitation did not reduce the DNA content.

In both, ammonium sulfate precipitation and acid precipitation, it is evident that there is a significant difference in the yield of α CD22scFv between clarified cell lysate and cell lysate. When cell free extract was subjected to acid precipitation, a α CD22scFv yield of $118.8\% \pm 21.8$ was reported, compared to yield of $87.5\% \pm 5.06$ in case of ammonium sulfate precipitation. The yield of α CD22scFv from pretreated clarified cell lysates was significantly different from that obtained from pretreated cell lysates ($73.12\% \pm 10.9$ for acid and $79.8\% \pm 10.9$ for ammonium sulfate). If 20-25% product loss during primary recovery is acceptable, the flexibility of operating the two pretreatment methods on cell lysate would be more economical as it allows cell debris removal and impurity reduction simultaneously with only one centrifugation step.

Table 2. Effect of pretreatment on residual DNA, chlorophyll, host cell protein, and α CD22scFv yield in algae extracts. Values given are averages from 3-5 replicates $\pm$ standard deviations.^{a,b,c,d,e} For each observation, means which do not share a common superscript letter are significantly different from the control no pretreatment ($p < 0.05$).

	Stage of primary recovery	Residual host cell protein (%)	Residual DNA (%)	Residual chlorophyll (%)	α CD22scFv yield (%)
Control	Clarified cell lysate	$100^a \pm 14.5$	$100^a \pm 9.12$	$100^a \pm 4.8$	$100^a \pm 16.3$
Ammonium sulfate (1M) precipitation at pH 8	Clarified cell lysate	$56.7^b \pm 6.7$	$100^a \pm 1.3$	$60.1^b \pm 6.3$	$87.5^a \pm 5.06$
	Cell lysate	$46.1^b \pm 7.4$	$100^a \pm 0.1$	$31.5^{ce} \pm 8.2$	$79.8^b \pm 10.9$
Acid precipitation at pH 4.5	Clarified cell lysate	$31.1^c \pm 9.3$	$91.7^a \pm 7.6$	$28.4^c \pm 14.7$	$118.8^a \pm 21.8$
	Cell lysate	$36.1^{bc} \pm 1.6$	$92.6^a \pm 10.5$	$55^b \pm 1.6$	$73.12^{bc} \pm 10.9$
Chitosan flocculation/precipitation at pH 5	Clarified cell lysate	$17.9^{cd} \pm 7.1$	$8.9^b \pm 7.9$	$1.37^d \pm 1.1$	$73.58^{bc} \pm 11.8$
	Cell lysate	$17.7^d \pm 4.9$	$14^b \pm 1.41$	$42.4^e \pm 0.2$	$65.3^c \pm 1.72$

In contrast to acid and ammonium sulfate precipitation, the lysate pretreatment with chitosan at pH 5 proved to be a better method in terms of overall impurities reduction. It not only reduced the

chlorophyll (58%) and protein (82%), but chitosan was able to flocculate the cell debris making their removal with low speed centrifugation possible. Most importantly, there was a significant reduction in DNA content to 14%, resulting in less viscous and easier to filter extracts. Chitosan pretreatment on clarified cell lysate showed even better results in terms of reduction of host cell protein (82%), chlorophyll (98%) and DNA (91%). The α CD22scFv yield (74%) was slightly better than the yield (65%) obtained by pretreatment of the cell lysate. The increase in yield may be attributed to reduction in loss of α CD22scFv bound to cell debris in case of cell lysate.

3.3. Effect of pretreatment of turbidity of pretreated extracts

All three pretreatment methods were able to decrease turbidity of centrifuged extracts (Figure 3). Ammonium sulfate and acid precipitation methods decreased turbidity of lysates 3- fold and 2- fold, respectively. The chitosan-mediated pretreatment resulted in more than tenfold decrease in extract turbidity. Lower viscosity of chitosan treated extracts along with the significant decrease in turbidity affected 0.45 μ m filtration efficiency significantly. We have observed a threefold increase in filtration throughput (processed volume / area) with chitosan pretreatment, compared to 1.5 fold each with ammonium sulfate and acid precipitation.

Figure 3. Effect of different pretreatment methods on turbidity (O.D.₇₅₀) of algae extract after centrifugation and before 0.45 μ m filtration. Values plotted are averages from 3 replicates (bars are standard deviations).

3.4. Combining pretreatment and a non-affinity capture step

To assess the potential benefit that extract pretreatment step had on the subsequent capture chromatography, we investigated the most logical pairing of pretreatment and subsequent adsorption step. Chitosan and acid pretreatment were paired with an anion exchange adsorption (Capto Q) and ammonium sulfate precipitation was paired with HIC using Phenyl Sepharose. The choice of anion exchange was dictated by the low isoelectric point (5.1) of α CD22scFv. Batch adsorption experiments revealed that α CD22scFv was completely adsorbed on Capto Q resin at pH 8 (lane 4 in 4b) from acid-pretreated extract (lane 3 in 4b) after reducing the extract ionic strength to less than 4 mS.

Figure 4. Purification of αCD22scFv after isoelectric precipitation and batch adsorption to Capto Q. (a) Coomassie-stained SDS-PAGE; (b) Western blot analysis of αCD22scFv samples using anti-FLAG-AP conjugated antibody. Lane 1: Molecular weight marker (kDa); Lane 2: Clarified cell lysate; Lane 3: Supernatant after isoelectric precipitation to pH 4.5; Lane 4: Supernatant after 30 minutes of binding with Capto Q resin at room temperature; Lane 5: Combined resin wash before elution; Lane 6: Elution fraction 1 (10.4 mS); Lane 7: Elution fraction 2 (14.8 mS); Lane 8: Elution fraction 3 (33.3 mS); Lane 9: Elution fraction 4 (62.2 mS); Lane 10: Elution fraction 5 (94.8 mS).

As indicated by the Western blot in Figure 4, the target protein eluted between the salt concentrations of 0.20 M and 0.40 M (10-33 mS/cm). Batch screening experiments with 1 M ammonium sulfate pretreated extracts showed that this pretreatment was not well-suited for Phenyl Sepharose resin capture step, since αCD22scFv did not bind significantly to this resin under tested conditions. In a separate experiment, we determined that for complete adsorption of FLAG-purified αCD22scFv more than 1.5 M ammonium sulfate would be required. As noted in Figure 2 above, 1.5 M ammonium sulfate concentration was not suitable for extract pretreatment because 50% of αCD22scFv was lost in the precipitate. Further optimization of the HIC step by changing adsorption pH and selecting more hydrophobic resin was not considered because HIC is typically low-yielding as a capture step and the other two pretreatments methods were compatible with ion exchange adsorption.

3.5. Capture chromatography of αCD22scFv from acid and chitosan pretreated extracts.

Acid and chitosan pretreated samples were analyzed by adsorption to and elution from HiScreen Capto Q resin using linear salt gradient.

Western blot and SDS-PAGE were carried out on eluted fractions (data not shown) to determine which fractions containing α CD22scFv should be pooled for quantification by FLAG affinity. The α CD22scFv pool was made of four fractions that eluted at conductivities between 13 and 20 mS/cm (Supplement Figure 1). About 10% of additional α CD22scFv was detected in fractions that were eluted between 20 and 33 mS/cm, but were not included because of the very low α CD22scFv concentration. The purity, yield and purification fold at each stage of the process is given in Table 3.

Table 3: Purity, yield, and purification fold at each stage of Capto Q purification process for chitosan acid pretreated extracts

	Chitosan precipitation			Acid precipitation		
Sample	Purity (% TSP)	Yield (%)	Purification fold	Purity (% TSP)	Yield (%)	Purification fold
Clarified cell lysate	0.08	100	1.0	0.12	100	1.0
Pretreated extract	0.25	88	3.0	0.39	85	3.0
α CD22scFv pool	1.7	40	20 (6.8)	1.6	26	12 (4.1)

In case of chitosan precipitation, purity increased 3-fold after the pretreatment and 6.8-fold after the anion exchange chromatography giving an overall purification fold of 20. In case of acid precipitation, purity increased 3-fold after the pretreatment and 4-fold after the anion exchange step – a total purification fold of 12. Interestingly, chitosan pretreatment yield of α CD22scFv from clarified cell lysate in this experiment was 88% - the high end of chitosan precipitation yields observed before. This experiment confirmed batch adsorption data and previously reached conclusion that both pretreatments would be compatible with anion exchange adsorption. We believe that “cleaner” extracts obtained with the chitosan pretreatment are the reason for greater overall yield i.e. 40% vs 26% for acid treated extract. The notable difference between the two experiments was the chlorophyll-stained resin after the adsorption of the acid-pretreated extract but not with the chitosan one. The low purity of α CD22scFv after the anion exchange capture step is a reflection of the low initial titer of α CD22scFv in cell lysates.

4. Discussion

This study investigated the process variables affecting extraction, recovery, and capture of recombinant protein in the algae extract. Composition of extraction buffer was optimized based on three factors - pH, NaCl (mM) and Tween. The maximum recovery yield of α CD22scFv was achieved at pH 8 with 400 mM salt and 0.5% Tween, the conditions that also maximize host cell protein (HCP), DNA, and chlorophyll extractability. A combination of low pH buffer, low ionic strength (low salt concentration) and absence of Tween in the lysis buffer could significantly reduce chlorophyll (70%) and total soluble protein (55%) concentrations; unfortunately, under those conditions a loss of 30% α CD22scFv was also observed. In conclusion, along with pH 8, high salt and Tween 20 are required for maximum recovery of chloroplast expressed α CD22scFv.

Investigation of different primary recovery methods, viz., ammonium sulfate precipitation, acid precipitation and cationic polymer (chitosan) precipitation on cell free extract as well as cell lysate of *C. renihardtii*, revealed interesting insights. The first important observation to make is that direct pretreatment of cell lysates was almost equally effective in reducing extract impurities as that of clarified extracts. For example, HCP was reduced 54% in cell lysate and 44% in clarified cell lysate by ammonium sulfate precipitation. Similarly, acid precipitation reduced HCP in cell lysates and cell free extracts by 64% and 69%, respectively. Ammonium sulfate was apparently more effective in reducing the chlorophyll content in cell lysates (68%) than in clarified extracts (40%), whereas acidic pretreatment exhibited the opposite effectiveness. The other important and relevant primary recovery criterion is the yield of the target protein. Ammonium sulfate precipitation and acid precipitation performed well with clarified cell lysate because almost 90% of α CD22scFv was recovered in the supernatants. However, with cell lysates, acid precipitation and ammonium sulfate precipitation resulted in α CD22scFv loss of 27% and 20%, respectively. A 15% loss of target protein during primary recovery is to be expected, and the observed greater loss in the presence of cell debris is not unreasonable. The exact reason for less than 80% α CD22scFv yield is difficult to give, but extracts

454 containing cell debris usually form large flocs, which enmesh recombinant protein and reduce the
455 observed yield.

456 The lysate pretreatment with chitosan at pH 5 proved to be a better method in terms of overall
457 impurities reduction. It not only reduced the chlorophyll (58%) and HCP (82%), but chitosan was able
458 to flocculate the cell debris making their removal with low speed centrifugation possible. Most
459 importantly, there was a significant reduction in DNA content to 14%, resulting in a less viscous and
460 easier to filter extracts. Chitosan pretreatment on clarified cell lysate showed even better results in
461 terms of HCP (82%), chlorophyll (98%), and DNA (91%) reduction

462 The observed loss of α CD22scFv with lysate (35%) and cell free extract (26%) during chitosan
463 precipitation at pH 5 was somewhat unexpected. The small size of α CD22scFv (28 kDa) and
464 flocculation pH near the pI of α CD22scFv should not have affected the protein yield that was
465 previously observed with cationic polymer precipitation of large proteins [28,14]. Therefore, possible
466 nonspecific interaction between the recombinant protein and chitosan, beyond the simple enmeshment,
467 might be the reason for lower than expected α CD22scFv yield. The 65% and 74% α CD22scFv yield,
468 respectively, during the chitosan pretreatment is below the desirable 85% primary recovery yield, but
469 potential benefit of significant DNA, chlorophyll, and HCP reduction to efficiency of capture
470 chromatography step could offset lower recovery yield.

471 The compatibility of pretreatment methods with the subsequent capture step was investigated by
472 screening two different chromatography resins. Chitosan and acid pretreated extracts followed by
473 anion exchange capture of α CD22scFv by Capto Q resulted in an overall purification fold of 20 and 12
474 respectively. Thus, anion exchange chromatography supplemented with the pretreatment methods on
475 algae extracts can have a synergistic beneficial effect on impurity reduction, filtration, and target
476 protein purity. However, extract pretreatment with 1 M ammonium sulfate was not optimal for direct
477 capture of α CD22scFv on Phenyl Sepharose because at least 1.5 M would be required to assure

complete protein adsorption. This conclusion may not be relevant for larger and more hydrophobic protein where 1 M ammonium sulfate concentration would be sufficient for direct HIC capture.

The pretreatment methods tested in this work have pros and cons, which depend on the target protein properties and pretreatment compatibility with potential capture methods. Pretreatment by acid and/or chitosan precipitation significantly reduces extract impurities and would be beneficial to any capture chromatography step as reduction in DNA, HCP, and chlorophyll pigments increases resin lifetime, prevent column compression, and decrease column fouling [13]. On the other hand, acid and chitosan precipitation require additional pH and ionic strength adjustment of pretreated extracts for efficient adsorption of the target protein on ion exchange resin. Overall, both these pretreatments coupled with anion exchange capture were successful in increasing the purification fold of the investigated recombinant protein. In short, the selection of a pretreatment method for conditioning of algae extracts should be chosen on a case-by-case basis. The comparison of the three precipitation methods and their compatibility with the following capture chromatography step on *C. reinhardtii* extracts lays down a road map for further testing and process development of the protein production platform.

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Author Contributions

PI, Zivko Nikolov conceptualized, developed and supervised the overall research plan. He also contributed to the preparation, review and editing of the manuscript. Neera Munjal designed and conducted experiments on the project involving α CD22scFv. She contributed to preparation and editing of the manuscript. Sayali Kulkarni and Katelyn Quinones contributed to batch adsorption and

502 chromatography data. All the authors contributed significantly to the discussions involving the
 503 research study.

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