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A scalable method to purify reflectins from inclusion bodies

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ABSTRACT

Structural proteins are an attractive inspiration for functional biobased materials. In nature, cephalopods skin colour modulation is related to the dynamic self-assembly of a family of structural proteins known as reflectins. To fully reach their potential as engineered bio-based materials, reflectins need to be produced by biotechnological means. One of the challenges is associated with establishing and optimizing reflectin purification processes to achieve the highest yield and productivity. Here, we studied purification strategies for two reflectin sequences from different organisms which were recombinantly expressed in a bacterial host at laboratory scale. Reflectins purification was then assessed by two chromatographic and one non-chromatographic methods. Methods were compared considering final purity and yield, productivity, cost and sustainability. The non-chromatographic method based on inclusion bodies washing presented the most promising results (protein purity > 90% and purification yields up to 88%). Our results contribute to define bioprocessing strategies to address the vision of biodegradable and sustainable protein-based materials.

1. Introduction

In an age where resources are limited and petroleum-based polymers are ubiquitous in our daily lives, natural polymers are promising alternatives due to their chemical, biological and mechanical diversity. Besides, these polymers are totally biocompatible and biodegradable [1]. Structural proteins are composed with genetic encoded amino acid sequences that can self-assemble into higher-order structures from nano, micro- to macro-scales [2]. They can be processed into a variety of formats namely films, fibers, foams, gels and particles, finding application in a wide variety of fields namely textiles, electronics and biomedicine [3,4].

A bottleneck to widely use protein-based polymers in materials science is their efficient and scalable production. Due to their specific characteristics (e.g. hydrophobic and intrinsically disordered characters), structural proteins are typically expressed in microbial hosts in high titres as inclusion bodies (IBs). The recovery of proteins from IBs requires high amounts of chaotropic agents, aqueous buffers and organic solvents, as well different consecutive unit operations, namely washes followed IBs recovery, metal-based tag and reverse-phase chromatography, as well as dialysis [2]. Thus, structural protein purification should be streamlined towards an integrated and continuous process with high productivity and low environmental impact [5,6].

Reflectins are proteins located in the skin and light organs of cephalopods, namely cuttlefish, squid and octopus. These proteins are involved in manipulating incident light in such a way that animals can modulate their colour modulation in milliseconds and camouflage [7]. Similarly, to other structural proteins, reflectins are characterized by highly conserved repeating amino acid motifs that alternate with variable linkers important for protein assembly and biological function [8,9]. Proteins from the reflectin family are easily expressed in bacterial host cells (200–1000 mg per liter) [10,11], which has facilitated their exploitation in a variety of fields. Reflectins perfectly interface with biological entities [12] enabling stem cell growth and differentiation

Abbreviations: IBs, Inclusion Bodies; IMAC, Immobilized metal affinity chromatography; RP, Reverse-Phase; IBP, Inclusion bodies purification; WB, Washing buffer.

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[13], or endowing new optical properties to human cells [14]. From a technological perspective, they are processed into mono- or multi-layered films and used as biophotonic devices actuated by mechanical or chemical stimulus [15], or as proton-conducting layers for bio-electronic devices [16].

Despite the enthusiasm and advances on the impact of native and engineered reflectin proteins, little attention has been paid to their bioprocessing, particularly to the optimization of downstream processes. Reflectins are intrinsically disordered proteins, which are expressed as inclusion bodies (IBs), due to their self-assembly propensity and high levels of cellular expression [17]. Therefore, the challenges are to maximize reflectin production while finding suitable ways to solubilize IBs and purify the protein without spontaneous precipitation or formation of aggregates [8,18]. In the literature, reflectin IBs were isolated and solubilized in buffers with one or both chaotropic agents: urea (4–8 M) and guanidine hydrochloride (0.75 - 7 M) [8,10,18–21]. Purification methods reported are based on chromatography using one or a combination of the following unit operations: immobilized metal affinity chromatography (IMAC), cation-exchange chromatography, or reverse-phase chromatography [8,10,18–21]. In general, the most common purification method used is IMAC or reverse-phase chromatography, being frequent the use of a second chromatographic step to increase the purity ratio (above 90%). However, increasing the number of consecutive unit operations decreases the yield and increases the process cost, which is undesirable for applications of proteins-based materials.

Here, we focused on optimizing reflectins production and on studying different purification strategies to achieve the highest productivity. We selected two reflectin sequences derived from distinct organisms, namely reflectin 1b from *Euprymna scolopes* [7] and reflectin 6 identified in *Octopus bimaculoides* [22]. After protein expression, we assessed two chromatographic methods – IMAC and Reverse-Phase chromatography – and one non-chromatographic method – IB washing – for purification. The purification strategies were compared and analyzed regarding yield, productivity, process costs, and water consumption. This research brings additional value to the field of bioseparation of structural proteins and accelerates the wide use of bio-based materials as alternatives to petroleum-based polymers.

2. Materials and methods

2.1. Materials

Buffers solutions were prepared using the following chemical compounds: Urea, Triton X-100, Tween-20, N-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) and tretadeutero acetic acid were purchased from Sigma-Aldrich. Guanidine hydrochloride (GndHCl), sodium dodecyl sulfate (SDS), acetic acid and dithiothreitol (DTT) were acquired from Fisher Scientific. Sodium chloride, Hydrochloride acid and Ponceau S were acquired from Panreac AppliChem. The other compounds were purchased in different suppliers: Sodium Hydroxide (VWR); Glycerol (ThermoFisher); Imidazole (Alfa Aesar); ethanol absolute (Merck Millipore); methanol (Honeywell); acetonitrile (Alfa Aesar); and Trifluoroacetic acid solution (Fisher Chemical). Reflectin genes were purchased from GeneCust (France). For cloning, *Escherichia coli* Nzy5α and Rosetta (DE3) competent cells were purchased from Nzytech (Portugal) and Novagen, respectively. Tris-base, Glycine, Luria Broth (LB) medium, ampicillin (Amp), kanamycin (Kan), chloramphenicol (Chlor), Agar, Yeast extract, Isopropyl β-D-1-thiogalactopyranoside (IPTG), and Tryptone were purchased from Nzytech (Portugal). All buffers and solvents used in this work for purification and their compositions are shown in Table S1.

2.2. Protein expression

Two proteins were used in this study: the full-length reflectin from *Euprymna scolopes* Reflectin 1b (R1b) (Uniprot entry: Q6WDN7) [7] and reflectin from *Octopus bimaculoides* coded as Ocbimv_skin_comp51140_c0_seq1_Scaffold210828 and deposited in Sequence Read Archive as BioProject PRJNA270931 [22]. For simplification, in this work, the reflectin from the octopus was called Reflectin 6 (R6). For R6 the N-terminal flanking region that comprises the first 17 amino acid residues, which is enriched in leucine was omitted for the gene synthesis. In addition, upon sequence alignment of 27 reported reflectins from different species this 17-aa length region was only detected in R6, meaning that it is a non-common sequence in reflectins. Sequences of both recombinantly expressed proteins are shown in supplementary information in Figure S1.

Genes of both proteins, R1b and R6 were synthesized in *E. coli* codon-optimized genes and purchased from GeneCust (France). Reflectin 1b gene was synthesized in a transport vector (pUC57) and then cloned into an expression vector (pET15b vector, Cat# 69661-3, Novagen) according to cloning protocols described in the methods section in the Supplementary Information. Reflectin R6 gene was already outsourced in pET15b allowing a final construct with an N-terminal Histidine tag.

R1b and R6 clones in pET15b (*ampR*) were transformed in *E. coli* Rosetta competent cells (*chlorR*) by the heat shock method (45 sec, 42 °C). Terrific Broth Medium (TB, composition 12 g/L tryptone, 24 g/L yeast extract, 0.4 % glycerol and sterile potassium phosphate buffer 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) was prepared in-house. The recombinant expression was conducted at laboratory scale. Briefly, from the glycerol stock 1 μL of bacteria were taken and inoculated in falcon tube with 10 mL of TB culture supplemented with 100 μg/mL Ampicillin and 30 μg/mL Chloramphenicol. Cultures were incubated at 37 °C overnight with constant orbital shaking (225 rpm). Next, 1 mL of the saturated cultures were added to 250 mL Erlenmeyer flasks with 100 mL of fresh TB media with respective antibiotics and grow for 4 h. Then 10 mL of cultures were inoculated into 2.5 L Erlenmeyer flasks containing 1 L of fresh TB media and grow at 37 °C with orbital shaking (225 rpm) until the culture reached an optimal density ($OD_{600\ nm}$) between 0.7 and 0.8. Reflectins expression was induced with 0.4 mM of Isopropyl β-d-1-thiogalactopyranoside (IPTG). After the induction, cells were grown at 30 °C overnight and then cells were harvested by centrifugation 4500*g*, 20 min at 4 °C. Cell extracts before (BI) and after induction (AI) were analyzed by 12.5% acrylamide SDS-PAGE gels to monitor reflectins expression.

2.3. Cell lysis

The harvested bacterial cells were resuspended in Lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5, added in a proportion of 4–6 mL per 1 g of the pellet) supplemented with cOmplete, EDTA-free protease inhibitor cocktail (cat# 11836170001, Roche, 1 tablet/ 50 mL of lysis buffer), and subjected to three cycles of freeze (−80 °C) / thaw (37 °C). Cell lysis was accomplished by supplementing the lysis buffer with 0.5 mg/mL chicken lysozyme and 5.0 μg/mL DNase I followed by incubation for 1 h at room temperature with gentle orbital shaking (20 rpm). In the case of non-chromatographic purification, lysis buffer was additionally supplemented with 10 mM EDTA. Subsequently, the lysed cell suspension was centrifugated (10956 *g*, 20 min, 4 °C) to fractionate and separate soluble and insoluble fractions. Fractions were analyzed with 12.5% polyacrylamide SDS-PAGE gel and reflectins were detected in the insoluble part.

2.4. Total protein concentration determination

The total protein concentration in all steps (after lysis, during washings, and purifications) was calculated with bicinchoninic acid (BCA) protein assay kit (cat# B9643, Sigma Aldrich) according to the supplier's protocol. Before quantification, it was necessary to remove the interfering substrates as well as solubilize the pellets containing reflectins. Therefore, protein-containing samples were precipitated with

cold ethanol. For this, the sample and cold ethanol were mixed in volume proportion 1:9, vortexed, and incubated for 1 h at −20 °C. Next, samples were centrifuged (10956 *g*, 20 min, at 4 °C) the supernatant was discarded and each pellet was solubilized by adding 5% (w/v) solution of SDS in 0.1 N of NaOH. The standard curve was prepared using the Bovine Serum Albumin (BSA) protein standard (cat#P0914, Sigma-Aldrich) and an identical procedure through BSA precipitation and solubilization in 5% (w/v) SDS in 0.1 N of NaOH. The working range of the standard curve was between 200 and 1000 μg/mL. All samples were prepared in triplicates in a Flat Transparent 96-well microplate from Sarstedt. Before absorbance measurements, 25 μL of each sample were mixed with 200 μL of BCA working reagent (50 parts of Reagent A (bicinchoninic acid in 0.1 N NaOH) with 1 part of Reagent B (Copper (II) Sulfate Pentahydrate 4% Solution)) and incubated at 60 °C for 15 min. The absorbance at 560 nm was obtained in Microplate Reader TECAN Infinite 200.

2.5. Chromatographic purification methods

As previously mentioned, both reflectins were found in insoluble fractions thus, before chromatographic purification it was necessary to wash these fractions to remove any soluble proteins, phospholipids and membrane proteins. Therefore, pellets containing reflectins were washed thrice with Washing Buffer 1 (WB1): 50 mM Tris-HCl,100 mM NaCl, 3 mM DTT, 5% glycerol, 1% Triton X-100, pH 7.5 to extract lipids and membrane-associated proteins. Next, the pellets were washed twice with WB2: 50 mM Tris-HCl,100 mM NaCl, 3 mM DTT, 5% glycerol, pH 7.5. Between washing, the centrifugation was performed (10956 *g*, 15 min at 4 °C). The final pellets containing reflectin proteins were resuspended and incubated overnight in solubilization buffer which was different according to the chromatography method: i) IMAC (SB): 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 5% glycerol, 7 M Urea, 5 M GndHCl, pH 7.5; ii) Reverse-phase chromatography (SBG): 50 mM Tris-HCl, 100 mM NaCl, 6 M GndHCl, 2.5% glycerol, pH 7.5.

2.5.1. Immobilized metal affinity chromatography (IMAC)

Purification was carried out in denaturing conditions and using the resin IMAC Sepharose 6 Fast Flow (cat# 17–0921-07, GE Healthcare, Cytiva) on a gravity flow system. The in-house packed resin (6 mL) was equilibrated with 5 column volumes (CV) of binding buffer (BB) supplemented with 30 mM of imidazole (BB composition: 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 2.5% glycerol, 30 mM imidazole, 2 M GndHCl, 4 M Urea, pH 7.5). The denatured proteins solution (typical concentration of 40–50 mg/mL of total protein) was diluted 1:2 with BB, loaded into a column and incubated with resin for an hour at room temperature and gentle orbital shaking (15 rpm). For R1b protein the resin was washed with 3 CV of BB. For the elution, imidazole concentration was increased to 300 mM to elute protein with 6 CV. The elution buffer (EB) was composed of 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 2.5% glycerol, 300 mM Imidazole, 2 M GndHCl, 4 M Urea, pH 7.5. For R6 protein the resin was washed with 3 CV of BB. Protein was eluted using a step gradient by increasing imidazole concentration in the EB (50 mM Tris-HCl, 500 mM NaCl, 2.5% Glycerol, 1 mM DTT, 5 M GndHCl, 4 M Urea, pH 7.5, and 100 mM, 300 mM, and 500 mM). The proteins were eluted with 2 CV per imidazole concentration for 100 and 300 mM, and the elution volumes were increased up to 4 CV with 500 mM imidazole.

After purification, elution fractions were pooled and loaded into the dialysis membrane Spectrum™ Pre-Treated with a cut-off 10 kDa and dialyzed against deionized water for 48 h with several changes of buffer (typically 7). The dialyzed solution was centrifuged (10956 *g*, 15 min, 4 °C) to separate the precipitated protein from the supernatant. Reflectin was removed from the tube walls with a spatula and was lyophilized.

2.5.2. Reverse-phase chromatography

Reverse-phase chromatography was carried out using HPLC Column Prep-C18, 100 Å, 10 μm, 250 × 30.0 mm (cat# 410910-302) in HPLC 1290 Infinity II system (Agilent Technologies). Reflectin crude extract was filtrated with a 0.22 μm PES filter and then loaded into the column. For R1b protein, the column was equilibrated with 25% solvent B (95% Acetonitrile, 4.9% High-Grade water (0.1% TFA): 75% of solvent A (99.9% high-grade water, 0.1% TFA) for 3 min. The protein was eluted with a linear gradient from 25% to 55% in 10 min at a flow rate of 25 mL/min (Figure SI 4 A). Finally, a washing step with constant condition 90% solvent B: 10% solvent A for 7 min and a re-equilibration with initial conditions. For R6 protein the column was equilibrated with 25% Buffer B (95% Acetonitrile, 4.9% High-Grade water, 0.1% TFA): 75% of Buffer A (99.9% high-grade water, 0.1% TFA) for 3 min. The protein was eluted with a linear gradient from 35% to 45% in 9 min and at a flow rate of 25 mL/min (Figure SI 4B). Finally, a washing step with constant condition 90% solvent B: 10% solvent A for 9 min and a re-equilibration with initial conditions.

2.5.3. Non-chromatography method

In this strategy, to recover protein with high purity ratio pellets containing reflectin proteins were washed with the solutions containing detergent (Triton X-100) and stepwise concentration increase of chaotropic salts (urea and/or GndHCl). More specifically, the buffers for the inclusion bodies washes were: i) Buffer W1: 50 mM Tris-HCl, 300 mM NaCl, 1 M Urea, 3 mM DTT, 5% Glycerol, 3% Triton-100, pH 7.5; ii) Buffer W2: 50 mM Tris-HCl, 300 mM NaCl, 2 M Urea, 1 M GndHCl, 3 mM DTT, 5 % Glycerol, 3 % Triton-100, pH 7.5; iii) Buffer W3: 50 mM Tris-HCl, 300 mM NaCl, 3 mM DTT, 5 % Glycerol, pH 7.5 to remove the detergent and chaotropic reagents. In each washing step, protein pellets were suspended in a buffer, and by using a tissue homogenizer pellets were reduced to small particles. After each homogenization, the suspension was incubated for 1 h with gentle orbital agitation (15 rpm) at room temperature to promote the gradual solubilization of less insoluble proteins, membrane proteins, and the removal of phospholipids lipids as well as other cell debris. During washings, the reflectin proteins stayed insoluble and were recovered by centrifugation (10956 *g*, 15 min at 4 °C). Finally, reflectin inclusion bodies were completely solubilized in a solubilization Buffer (SB: 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 5% glycerol, 7 M Urea, 5 M GndHCl, pH 7.5) for 48 h at room temperature and constant orbital shaking (15–20 rpm) and, then dialyzed for 48 h against water and lyophilized.

The productivity of the purification methods was calculated according to equation (1):

$$productivity = \frac{mass\ of\ pure\ protein\ purified\ from\ 1L\ of\ cell\ culture(\frac{mg}{L})}{number\ of\ days\ needed\ to\ obtain\ pure\ protein} \quad (1$$

2.6. Reflectins characterization

2.6.1. Western-blot

Purified reflectins were analyzed by Western Blot by loading 10 μg of protein into 12% polyacrylamide SDS-PAGE gel run (120 V for 1h30min) Next, the transfer from a gel to a Nitrocellulose Membrane with 0.45 μm pore size (Cat# 1620115, Bio-Rad) using Mini Trans-Blot® Cell Biorad system, using an optimized transfer buffer (25 mM Tris, 192 mM Glycine, 20% (v/v) Methanol) and transfer conditions of 200 mA for 30 min. The membrane was washed twice, after the transference with TBS buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl). As a blocking solution was used 3% BSA (Bovine Serum Albumin) (w/v) was in a TBS buffer for 1 h at room temperature. Anti-His conjugate HRP antibody with the dilution 1:2000 (cat# 1014992, Qiagen,) was used for the detection overnight. Finally, the membrane was washed twice with 0.1 % Tween-20 in TBS buffer and once with TBS buffer. For detection, was used 1-Step-TMB blotting (cat#34018, ThermoScientific) as a colorimetric substrate. The blot results were recorded in a ChemiDoc XRS+ (BioRad).

2.6.2. N-terminal sequencing

The reflectins purified samples were analyzed through N-terminal sequencing to confirm the protein sequence expressed and purified. For the preparation of the sample, 10 μg of R6 was loaded in a 12.5% acrylamide SDS-PAGE gel and then transferred to a membrane (PSQ PVDF, 0.22 μm (cat# ISEQ00010, Millipore)) was performed using electroblotting buffer: 10 mM CAPS, 10% (v/v) Methanol pH 11 for 30 min at 50 V. The membrane was stained using a Ponceau solution (1% (w/v) Ponceau in 1% (v/v) Glacial Acetic Acid). The membrane was air-dried, protein bands were and analyzed at ITQB (Oeiras, Portugal), using an ABI Procise Protein Sequencer with an ABI Microgradient Pump System, and an ABI Programmable Absorbance Detector.

2.6.3. MALDI-TOF mass spectroscopy

The molecular weight of purified reflectins was analyzed by MALDI-TOF Mass Spectroscopy (MS) at ITQB (Oeiras, Portugal). To prepare the sample, the protein solution was desalted and concentrated using POROS C8 (Empore, 3 M) and eluted directly on the plate with 1 μL of 10 mg/mL Sinapic acid (Sigma) in 50 % (v/v) acetonitrile and 5 % (v/v) formic acid (LC/MS grade, Fisher). Data were acquired in Linear High Mass Positive mode using a 5800 MALDI-TOF/TOF (ABSciex) mass spectrometer and TOF/TOF Series Explorer Software v.4.1.0 (ABSciex). The raw MS data were analyzed using Data Explorer Software v. 4.11 (ABSciex). External calibration was performed using Protein MALDI-MS Calibration Kit (MSCAL3, ProteoMass).

2.6.4. Circular dichroism spectroscopy

Reflectins secondary structure was determined by Circular Dichroism (CD) spectroscopy. Both reflectins were dissolved in 5% (v/v) Acetic Acid pH 2.0 and the final concentration was determined by BCA assay using the same method as described before. The spectra were acquired at 25 °C using the wavelength between 190 nm and 260 nm with a step size and bandwidth of 1 nm. Triplicates of each sample were measured, and it was recorded three times for each sample, the accumulations were averaged and smoothed. The CD analysis was performed at BioLAB (UCIBIO, FCT-NOVA). The secondary structure content was calculated and analyzed through the BeStSel web server [23].

2.6.5. Attenuated total reflectance- fourier transform infrared spectroscopy (ATR- FTIR)

Reflectins lyophilized powder was solubilized in 5% tetra-deutero acetic acid pH 2.0 and then lyophilized to be analyzed through ATR-FTIR. Spectra were recorded using an adaptor in a Spectrum Two FTIR Spectrometer (Perkin Elmer). The range of spectra recorded was between 4000 and 400 cm^{-1} in a total number of 25 scans. Background air was performed, and samples' spectra were recorded under a force gauge of 83. Secondary structure assignment is possible by analyzing the peaks in the amide band I region (1600–1700 cm^{-1}). Further, amide bands were deconvoluted using OriginPro 2023 software and the Peak Deconvolution tool from Origin. The automatic second derivative method in the software was used to identify peak positions and the smoothing method used was quadratic Savitzky-Golay (second polynomial order) with 20–25 points. All bands were fitted to determine the relative abundance of secondary structures.

3. Results and discussion

3.1. Sequence selection & research strategy

We began our studies by selecting the reflectin sequences. To increase variability we chose two reflectin sequences from different cephalopod species and animal tissues: Reflectin 1b (R1b) identified in the light organ reflector of *Euprymna scolopes* [7], and Reflectin 6 (R6) found in the skin tissue of *Octopus bimaculoides* [22]. While the expression and purification of R1b has been described in the past [21], our work reports for the first time the recombinant expression, purification and characterization of R6. Both proteins possess the characteristic domains found in reflectins [7] (Fig. 1A), namely highly conserved repeating motifs (RMs) (R1b and R6 contain three and four respectively) that comprise the sequence $M/FDX_5MDX_5MDX_{3\text{-}4}$ (where X = S, Y, Q, W, H, R, G), and a highly conserved N-terminal domain MEPMSRMTMDFQ/HGRY/LMDSQGRM/IVDP. All motifs are connected by linkers with less conserved length and composition. Following protein selection, we proceed with recombinant expression, purification, and characterization (Fig. 1B).

3.2. Recombinant production of reflectins

To maximize the expression yields of R1b and R6 in Rosetta *E.coli* cells, we tested two different growth media (Luria Broth (LB) and Terrific Broth (TB)) and compared them in terms of bacteria pellet yield. Thus, with identical expression conditions (time, temperature, agitation, and inducer concentration (0.4 mM IPTG)) we observed a four-fold increase in bacteria pellet in TB media when compared with LB media (LB: 3.8 ± 0.8 g of wet cells / L of culture, while TB: 12.4 ± 1.9 g of wet cells / L of culture). This is an expected result since despite both culture media being commonly used, TB contains slightly more tryptone and almost five fold higher amounts of yeast extract than the standard LB media. This results in a higher yield of bacteria pellets. Another important difference is the carbon source, as in LB media bacteria use catabolized amino acids, while in TB media the carbon source is glycerol, which helps to significantly increase bacteria's lifetime [24].

Regarding protein production, histidine-tagged R1b and R6 were successfully expressed after induction with IPTG (Fig. 2A). In accordance with the literature, R1b is over-expressed as inclusion bodies (IBs) [14,19,25]. In the case of R6, it was for the first time reported the recombinant expression of this protein, and similar to R1b it was expressed in IBs. Using the *E.coli* expression system we achieved high expression yields, on average: 314 ± 118 mg/L and 716 ± 161 mg/L for R1b and R6 respectively (Fig. 2B).

The production of a two-fold higher mass of R6 in comparison to R1b could be related to the difference in bacterial pellet yields. When expressed in TB media we obtained around 1.5 times lower mass of the bacterial cells for R1b in comparison to R6 (8.3 ± 1.3 and 13.5 ± 2.0 g of wet cells / L of culture respectively).

3.3. Reflectins purification

Following protein selection and recombinant expression, we proceeded with purification using two chromatographic methods previously reported in the literature - immobilized metal affinity chromatography (IMAC) [26,27] and reverse phase (RP) chromatography [10,28] – and proposed a non-chromatographic method based on IBs washing (Fig. 3). It should be noted that before purification with chromatographic methods, the obtained IBs need to be cleaned. This process includes several washing steps with buffers containing non-ionic detergents (e.g. Triton X-100, Tween-20). This step allows the removal of cell debris and phospholipids from the cell membrane, as well as membrane proteins and soluble proteins that remained in the pellets after the fractionation step. Following the washing step, IBs were solubilized in a solubilization buffer with high concentrations of chaotropic agents (7 M of urea or 6 M guanidinium hydrochloride), after that was loaded on the chromatographic columns.

3.4. Immobilized metal affinity chromatography (IMAC)

Both reflectins R1b and R6 were purified using IMAC under denaturing conditions, then dialyzed against water to remove all salts, and finally lyophilized. The total protein mass and reflectins mass were monitored along the process. During purification, we detected the loss of a significant amount of reflectin, in particular between pellet solubilization and lyophilization, as demonstrated in Table 1. In fact, in IMAC

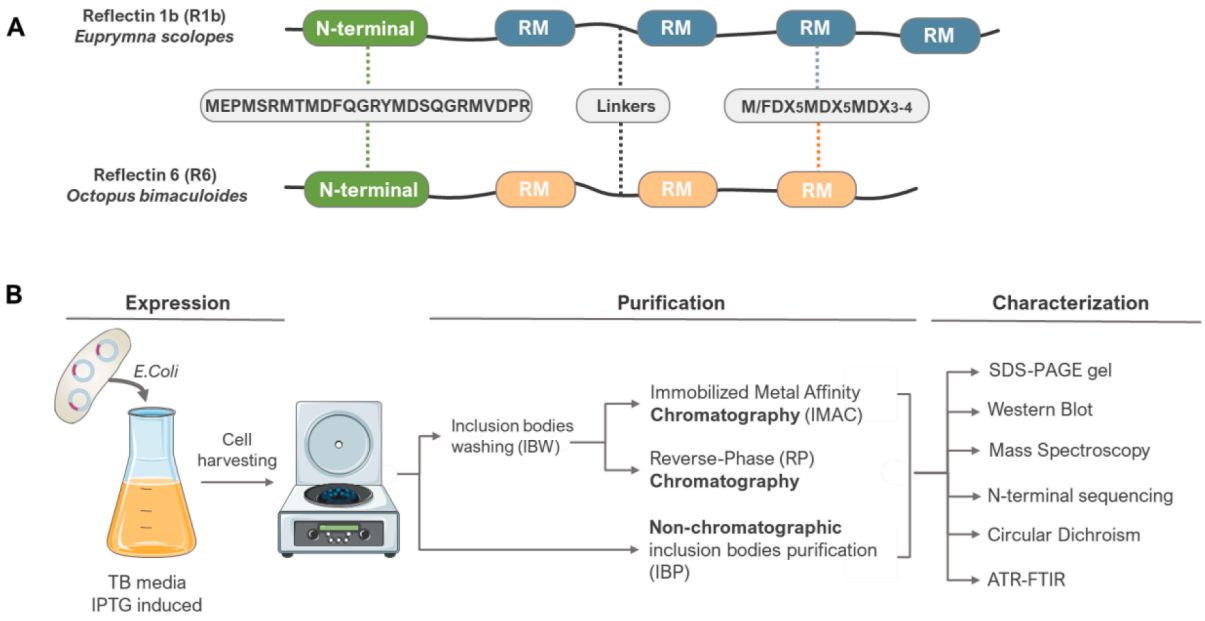


Fig. 1. A) Schematic representation of the typical reflectin sequence structure, with displayed conserved N-terminal motif (N-terminal), repeating motifs (RM), where X = S, Y, Q, W, H, R, G and linkers with variable sequences. B) Schematical representation of the research strategy conducted in this work: including reflectin expression and purification methods, as well as biophysical and characterization techniques.

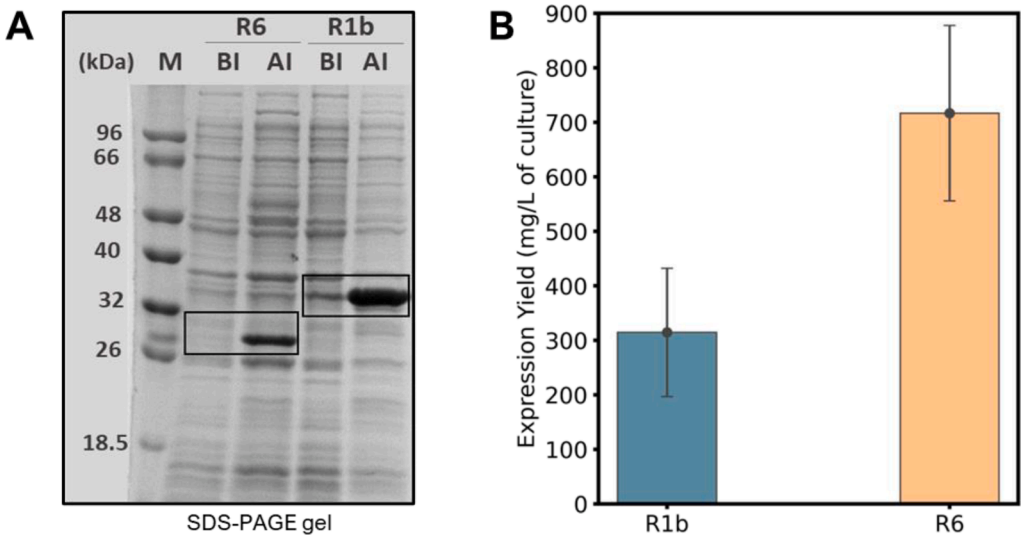


Fig. 2. Analysis of the recombinant expression of histidine-tagged reflectins in TB media. A) SDS-PAGE 12.5% gel of crude extracts obtained after expression at 30 °C for R1b (MW 38.5 kDa) and R6 (MW 31.5 kDa). Expression was induced with 0.4 mM IPTG. Samples were collected: before induction (BI) and after overnight expression (AI). The marker lane (M) contained the Low Molecular Weight marker (Nzytech). The gel was stained with Coomassie blue. The black box highlights the bands corresponding to the R1b and R6 monomers. B) The average expression yields of reflectins (n = 3). The total protein concentration of the insoluble fraction was calculated using BCA assay and reflectins mass was estimated based on the densitometric analysis in ImageLab software (Biorad) of the reflectins bands in the SDS-PAGE gel run after fractionation.

purification, the average total protein amount loaded into the column was optimized to approximately 25 ± 7 mg/mL of resin. This amount is much lower than the dynamic binding capacity of the resin which according to the supplier is ≈ 40 mg His-tagged protein/mL medium (in Ni^{2+} charged medium). Moreover, the concentrations of the used buffer components (e.g. 50 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, 2.5% Glycerol) and chaotropic agents (4 M urea and 2 M GndHCl) were lower than the maximum compatible concentrations reported by resin supplier (100 mM Tris-HCl, 1.5 M NaCl, 5 mM DTT, 50% Glycerol, 8 M Urea, and 6 M GndHCl). Thus, the resin overload or decrease of the dynamic binding capacity could not be the major reasons that lead to protein loss. Furthermore, as shown in Figure S2 during purification reflectins were eluted upon imidazole increase, while flow-through and washing fractions contained mainly contaminant proteins.

Note: crude calculations were performed with reflectin-containing solution after solubilization: Total insoluble protein mass (mg) corresponds to total mass of protein found in insoluble fraction after expression in one liter of culture and quantified by BCA assay; Initial reflectin content (%) represents reflectin content estimated by SDS-PAGE gel densitometry; Estimated reflectin mass (mg) is reflectin mass produced in one liter of culture calculated from estimated reflectin content × total protein mass. For the purified section: Total lyophilized mass (mg) is the weight of total lyophilized mass after purification; Total protein mass (mg) corresponds to the total protein mass in lyophilized powder quantified by BCA assay; Estimated reflectin content (%) represents the reflectin purity estimated by SDS-PAGE gel densitometry; Estimated reflectin mass (mg) corresponds to reflectin mass in lyophilized powder calculated from estimated reflectin content × total protein mass. Yield = (Estimated Purified Reflectin Mass / Estimated Crude Reflectin Mass) × 100.

An additional possible cause could be the incomplete solubilization of protein pellets. After overnight solubilization of the washed IBs, the resultant solutions were turbid with small insoluble particles, visible to the naked eye. Thus, to avoid column clogging the protein solutions

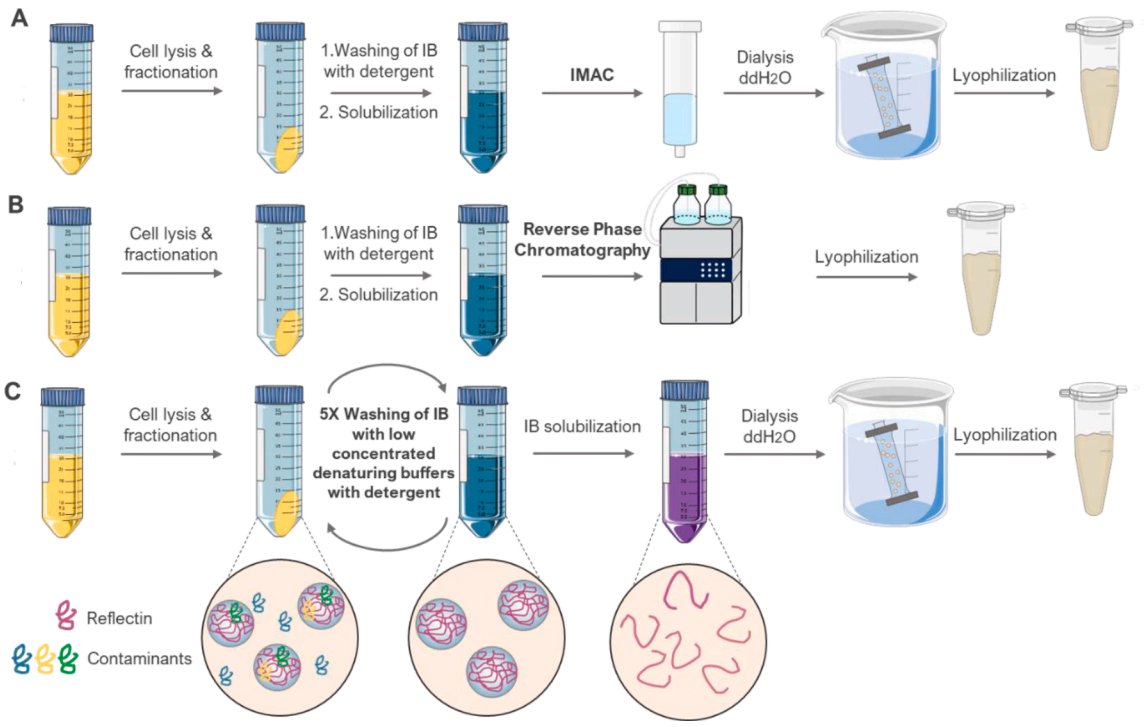


Fig. 3. Schematic representation of the workflow for each chromatographic and non-chromatographic purification method tested in this study: A) Immobilized metal affinity chromatography (IMAC); B) Reverse-Phase chromatography; C) Purification of inclusion bodies (IBs) through washings with low-concentrated denaturing buffers.

Table 1
Evaluation of reflectins purification through IMAC.

Protein extract	Criteria	Total Protein	R1b	Total protein	R6
Crude	Total insoluble protein mass (mg)	550 ± 55	–	892 ± 124	–
	Initial reflectin content (%)	–	80 ± 3	–	66 ± 4
	Estimated Reflectin mass (mg)	–	430 ± 44	–	585 ± 87
Purified	Total lyophilized mass (mg)	168 ± 1	–	195 ± 1	–
	Total protein mass (mg)	166 ± 3	–	195 ± 5	–
	Estimated reflectin content (%)	–	95 ± 2%	–	89 ± 3%
	Estimated reflectin mass	–	158 ± 2	–	173 ± 4
	Purification yield (%)		37 ± 2%		29 ± 4%

were centrifuged at high speed and were filtered before loading them onto the IMAC column. Further analysis of the pellets after centrifugation, revealed that their major fractions (from 60 to 70% calculated by densitometric analysis of SDS-PAGE gels) consist of non-solubilized reflectin proteins. These pellets may result from the non-complete solubilization of IBs that may require longer incubation time (e.g. 48 h instead of overnight) or even high energy input (e.g. heating of the solution) [29]. Moreover, the presence of the non-lysed bacterial cells in the pellet may also affect the overall purification yield, since for its calculation we used the total amount of reflectins that were expressed and not the amount that was solubilized prior to purification.

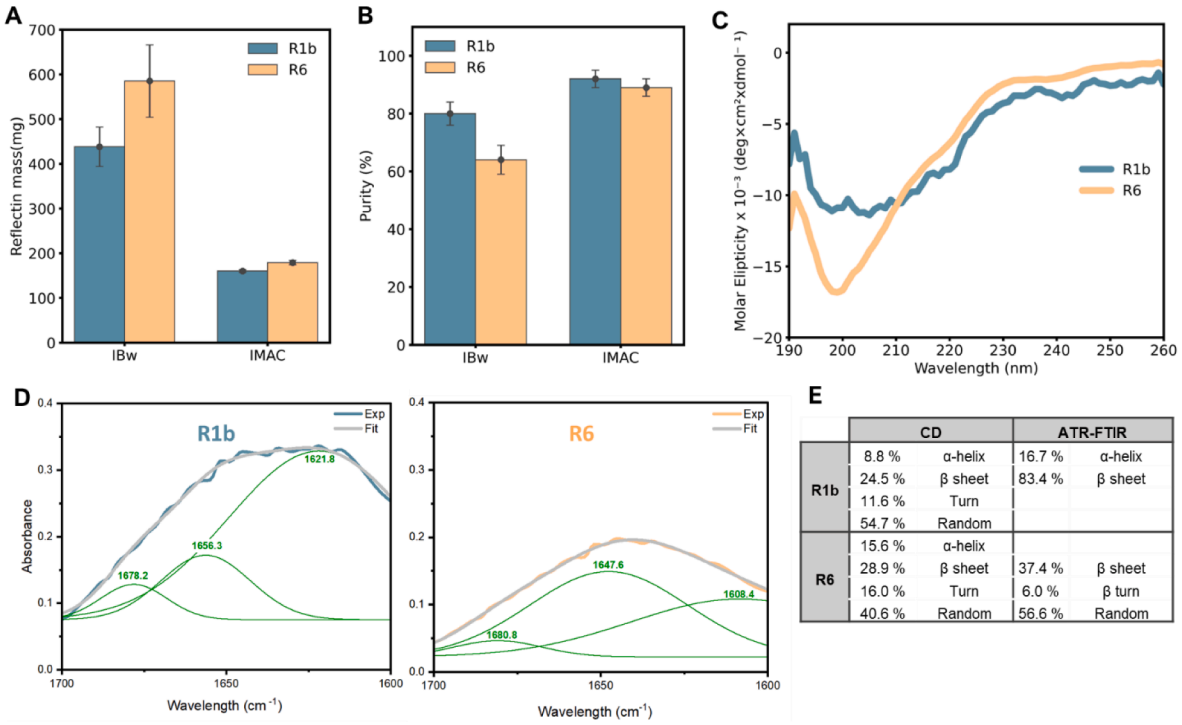
In the end, the IMAC purification process yielded approximately 158 ± 2 and 173 ± 4 mg of protein per L of culture for R1b and R6, respectively (Fig. 4A). According to our results shown in Fig. 4B the purity of proteins was improved from 80% up to 92% for R1b and from 62% up to 89% for R6, when we compare the crude extract with the final purified protein.

To confirm the identity of reflectins, namely through the presence of His-tag sequences, we performed N-terminal sequencing (Figure S3) and western-blot analysis (Figure S4). Additionally, the protein's molecular weight was validated by MALDI-TOF where peaks corresponding to the His-tagged reflectins monomers were observed. The following monoisotopic masses were detected: m/z 38402.5 Da for R1b and m/z 31462.4 Da for R6 samples and are close to the estimated ($MW_{calculated}$ = 38559.8 Da and 31500.1 Da respectively in webtool ProtParam [30]) (Figure S5).

Purified proteins were further characterized by CD and ATR-FTIR spectroscopies, showing that both proteins contain a mixture of secondary structures in agreement with their intrinsically disordered propensity previously reported [18,31]. The R1b solution (Fig. 4C and E) showed α-helix, β-sheet, and disordered conformations (minimum negative peaks at 208 nm, 218 nm, and 219 nm respectively). The deconvolution of R1b ATR-FTIR spectra obtained for a lyophilized protein sample (Fig. 4E) showed a higher percentage of β-sheet structures (83.4%), an effect observed for other silk proteins upon water removal [32]. Regarding reflectin R6, CD analysis indicated a high percentage of disordered moieties (maximum negative peak ~ 200 nm), with the presence of some β-sheet organization (small peak ~ 219 nm). The deconvolution of the R6 ATR-FTIR spectra also indicated that > 56% of protein is found in disordered conformation.

3.5. Reverse-phase chromatography

Reverse-phase chromatography has been successfully used to purify reflectin proteins from *Doryteuthis pealeii* [11,34,35]. Ordinario and colleagues obtained high yield (0.8 – 1 g/L of culture) with high purity (>99 %) [35] using this chromatographic method. Thus, we attempted the purification of R1b and R6 by reverse-phase chromatography. In our hands, this method provided very low protein yields (Fig. 5A), recovering 27 ± 5 mg for R1b and 29 ± 8 mg for R6. This is 5 to 6 times lower



	CD		ATR-FTIR	
R1b	8.8 %	α-helix	16.7 %	α-helix
	24.5 %	β sheet	83.4 %	β sheet
	11.6 %	Turn		
	54.7 %	Random		
R6	15.6 %	α-helix		
	28.9 %	β sheet	37.4 %	β sheet
	16.0 %	Turn	6.0 %	β turn
	40.6 %	Random	56.6 %	Random

Fig. 4. Analysis of reflectins purification through IMAC. A) Analysis of the R1b and R6 mass at two different steps: after IB washing with buffer containing detergent, and after IMAC and lyophilization (IMAC). Reflectin mass was estimated through BCA quantification and densitometry. B) Reflectins' purity variation different purification steps. C) Far-UV spectra for R1b and R6 dissolved in 5 % (v/v) acetic acid solution, pH 2.0. D) Absorption spectra and deconvolution of the powder R1b and R6 amide l bands. E) Summary of the secondary structure prediction after CD spectra analysis through BestSel [23] and deconvolution of the amide l region of the ATR-FTIR spectra in OriginPro Version 2023 software [33].

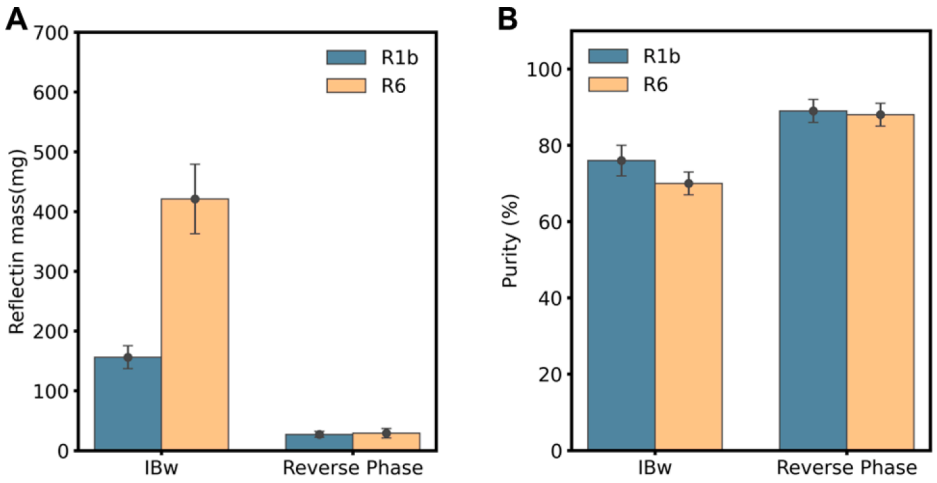


Fig. 5. Analysis of reflectins purification through reverse phase chromatography. A) Analysis of the R1b and R6 mass at two different steps: after washing of IBs with buffer containing detergent compared with after reverse-phase chromatography purification and lyophilization. Reflectins mass was estimated through BCA quantification and SDS-PAGE gel densitometry analysis using Image Lab software (Biorad). B) Reflectins' purity variation in different purification steps.

than the amount recovered by IMAC purification. Additionally, the purity of protein fraction was improved, but it still was lower than 90% (Fig. 5B, 89% for R1b and 88% for R6).

These significant protein losses and lower purity of the final fractions are related to the incomplete protein solubilization before loading, as it was previously discussed for IMAC purification. In addition, we observed an abnormal reflectin elution pattern with several intense peaks at variable retention times during chromatographic runs (Figure S6). All peaks were collected in separate fractions and analyzed by SDS-PAGE gels (Figure S6), showing that reflectins were eluting throughout the run with different retention times and peak profiles. Such unusual elution behavior for both R1b and R6 can be explained by the disordered and dynamic conformations characteristic of IDPs. As a result of these protein sequence properties, reflectins can display different hydrophobic domains that will interact differently with the solvent and the chromatographic matrix causing variations in the retention time [36].

3.6. Inclusion bodies washing purification

Although IMAC and reverse-phase chromatography could provide reflectin-enriched fractions, these two methods provided low

purification yields (below 38% and 17% respectively), are time-consuming, and use high amounts of water, metals, solvents (acetonitrile and TFA). Thus, we explored an alternative method based on IBs washing with buffers containing denaturing compounds in combination with detergent (as shown in Fig. 3C).

The highly insoluble nature of IBs enables the use of buffers containing low concentrations of the chaotropic agents (e.g., 0.5–2.0 M of urea and/or 0.5–1.0 M of GndHCl) to remove the contaminants without dissolving and losing the protein of interest.

Since IBs washing purification (IBP) protocol is mostly based on IBs pellet washing followed by centrifugations, it was necessary to improve cell lysis and reduce the amount of non-lysed bacterial cells. As we referred previously, this was critical for the purification yield in chromatographic methods. To improve cell lysis, we used a different lysis buffer (50 mM Tris- HCl, 100 mM NaCl, 0.5 mg/mL lysozyme, 5 μg/mL DNase, 10 mM EDTA pH 7.5 supplemented with protease inhibitor in proportion 1 tablet/ 50 mL cell extract). The addition of EDTA into the lysis buffer targeted the increase in the susceptibility of *E.coli* cells to lysozyme that hydrolyses by disintegrating the outer membrane of gram-negative bacteria [37,38]. Moreover, we increase the time of IBs solubilization from overnight incubation to 48 h to ensure complete denaturation and dissolution of reflectins. As the result, we obtained 257 ± 10 mg and 837 ± 15 mg for R1b and R6, respectively (Fig. 6A). Additionally, the purity of reflectins fractions increased from 80% to over 93%, as demonstrated in Fig. 6B and C. We evaluated the secondary structure of purified proteins and as shown in Fig. 6D the shape of the curves was identical to the one observed for reflectins purified by IMAC (Fig. 4D).

3.7. Comparison between chromatographic and non– chromatographic methods

The three purification methods – IMAC, reverse-phase chromatography, and IB washing purification – were compared in terms of purification yield, time, productivity, buffer, reagent consumption and costs. Purification yields differ significantly between the processes (Fig. 7A) with the non-chromatographic method showing the greatest values for reflectins purification. As previously discussed, the incomplete solubilization of IBs-containing pellets could contribute to the significant protein loss and low purification yields of IMAC and reverse-phase chromatography. By improving bacterial cell lysis and increasing the time of reflectins dissolution, during IB washing purification, we were able to completely solubilize IBs and increase the mass of recovered reflectins.

Such optimization can be easily implemented before reflectins purification when using the chromatographic methods and certainly will improve purification yields. Nonetheless, it will require additional steps after cell lysis to remove EDTA prior to sample loading into IMAC column to avoid undesirable metal stripping and protein sample contamination. One of the most important advantages of IB washing purification is the absence of the stationary phase, which many times limits the productivity of the purification process and significantly increases its costs. On the other hand, during IB purification all washing steps are made in a 50 mL tube. As a result, the IB purification that uses buffers with denaturing agents at low concentrations, is the method with the highest productivity for both reflectins tested in this work. Besides, when we compare this strategy for both proteins, R6 showed higher productivity which is mostly related to the highest bacterial and expression yields for this protein in comparison to R1b.

Additional comparison of various parameters for each purification

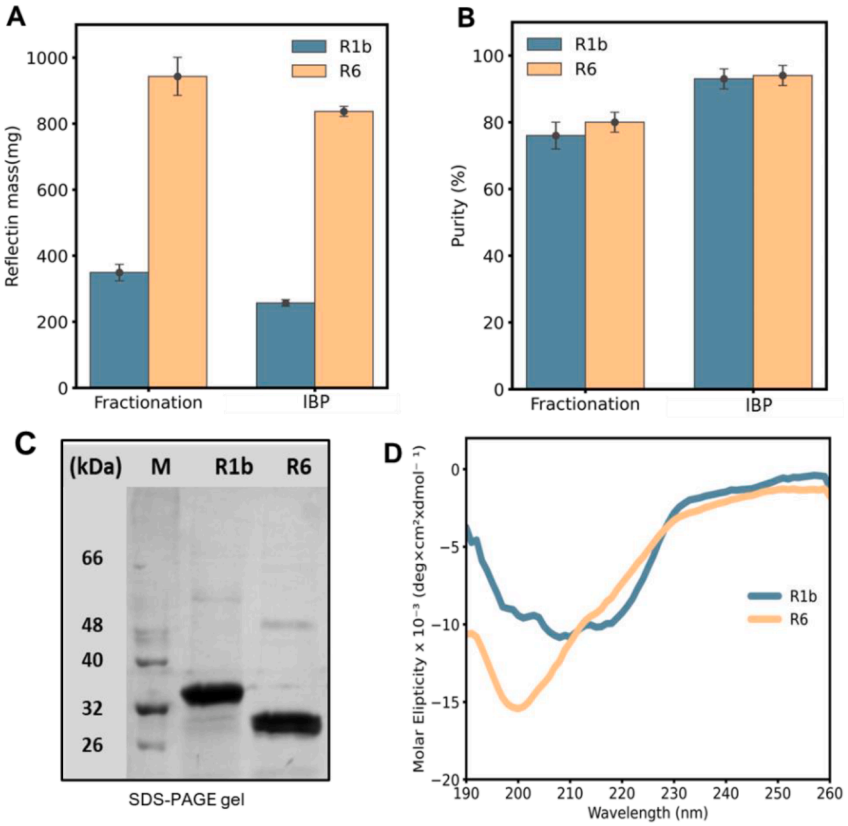


Fig. 6. Analysis of IB washing purification (IBP). A) R1b and R6 mass at two different steps: after cell fractionation and after IB washing purification and lyophilization. Reflectins mass was estimated through BCA quantification and densitometry. B) Reflectins purity variation in different purification steps (estimated through densitometry). C) Analysis of purified R1b and R6 by SDS-PAGE. D) Far-UV spectra for R1b and R6 dissolved in 5 % (v/v) acetic acid solution, pH 2.0.

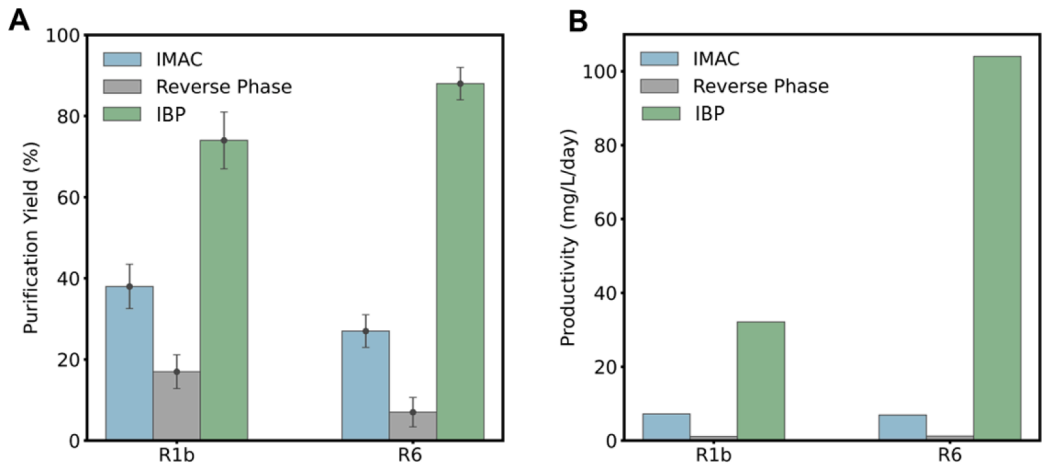


Fig. 7. Comparison of the purification yields (A) and productivity (B) for different purification methods and both reflectins R1b and R6.

method is shown in Table 2. IB purification showed several important advantages when compared to the chromatographic methods. Regarding water consumption, although the volume of water used is high due to the dialysis step it is still lower when compared to the IMAC. Contrary to reverse-phase chromatography, there is no need to use any organic solvent during IB washing purification. Additionally, in the IMAC the binding and elution buffers contain imidazole and metals (e.g Ni^{2+}, Co^{2+}, Zi^{2+}, Cu^{2+}) immobilized in the resin. This generates wastes with metal ions due to the ions stripping for resin regeneration and reuse. In some cases, due to the instability of the chelate-ion complex in purification conditions metal ion leakage may occur and contaminate the eluate.

Regarding buffer costs and reagents consumption, reverse phase chromatography is the most expensive followed by IMAC. Although reverse phase chromatography does not require significant buffer usage, it presents the lowest protein recovery yields (17% and 7% for R1b and R6 respectively). Thus, to obtain 1 g of pure recombinant reflectin there is the need to express more protein and consequently higher volumes of buffer are needed to wash the IBs and to solubilize them. On the other hand, because of high purification yields (74% for R1b and 88% for R6), IB washing purification showed the lowest buffer costs and reagent consumption. Consequently, the application of this method for reflectins purification is more sustainable since will generate less waste.

When we analyzed the number of days required to purify 1 g of protein by IB washing purification, it is almost three times lower when compared to the other processes. This considerable difference is mostly related to the low purification yield of the chromatographic processes, as well as limitations related to the stationary phase binding capacity. Therefore, IB washing purification presents the highest productivity values as shown in Fig. 7B.

Although studies described here were performed in the laboratory scale, all steps of the IB purification method are scalable, which will enable to generate an efficient and economical bioprocess for reflectin production. There are five major steps involved in reflectins production and purification: i) protein recombinant production, ii) cell lysis, iii) IBs purification, iv) IBs solubilization, and finally v) dialysis and lyophilization. The recombinant production of reflectin can be adapted to *E.coli* fermentation in a bioreactor. Cell lysis for IBs isolation can be performed through several mechanical or chemical methods. Due to accessibility of equipment and reagents, in this work we combined freeze–thaw with lysozyme. However, for industrial scale freeze–thaw is not ideal since it can be time consuming and is not highly effective. Nonetheless, studies have shown that the combination of sonication and lysozyme results in highly efficient IBs isolation methods, they cause very effective bacterial cell disruption and can be used at large scale [39]. The washings and solubilization of the IBs can be performed in flask with constant

Table 2
Evaluation of different parameters of chromatographic and non-chromatographic methods tested and comparison between them.

Purification method		**IMAC**		**RP Chromatography**		**IB purification**	
Protein ID		**R1b**	**R6**	**R1b**	**R6**	**R1b**	**R6**
Stationary phase (resin/column) volume and price (€)		Fast flow Sepharose 6 resin: 25 mL – 262€		C18 column: 6000€		Not applicable	
Buffer costs (€ / g of recombinant reflectin)		160.0	165.0	177.0	170.0	62.0	27.5
Amounts of buffer reagents	Tris (g)	21.2	24.0	18.9	16.3	5.4	2.3
	NaCl (g)	20.2	23.0	18.2	16.0	16.0	12.0
	DTT (g)	9.8	9.3	15.0	12.4	32.0	1.6
	Urea (g)	618.0	814.0	219.0	190.0	82.0	38.0
	GndHCl (g)	511.0	666.0	248.0	240.0	59.2	24.0
	Imidazole (g)	20.8	31.8	–	–	–	–
	Glycerol (mL)	106.0	100.0	79.0	67.0	45.0	17.6
	Triton (mL)	7.0	5.6	16.0	13.5	17.5	10.6
Organic solvents: volume (L) and price (€)/50 injections in HPLC		Not applicable		Acetonitrile: 16.3 L = 694.5 €		Not applicable	
Water for purification (L)		Buffers: 6.0 L Dialysis: 80.0 L Total: ~86.0 L		Buffers: 0.5 L HPLC: 20.0 L Total: ~20.5 L		Buffers: 2.0 L Dialysis: 50.0 L Total: ~52.0 L	0.6 L 50.0 L ~50. 6L
Purification yield (%)		38.0	27.0	17.0	7.0	74.0	88.0
Purification time (days / g of recombinant protein)		22	23	24	25	8	8
Productivity (mg / L.day)		7.2	6.9	1.0	1.2	32.0	104.0

magnetic stirring to ensure uniform suspension. Also, the dissolution of the reflectin IBs can be adapted to a continuous system [40]. To make the process more environmentally friendly the detergent Triton X-100 should be replaced by more sustainable non-ionic detergents such as Tween 20. However, since the detergent capacity of Tween 20 is lower [41] some optimizations in the protocol need to be performed (e.g. longer washing time, more washing steps, higher detergent concentration). Finally, to remove salts and denaturing agents the dialysis can be replaced by ultra-diafiltration or crossflow ultrafiltration [39,42] that will reduce drastically the water and buffer consumptions, making the process even more sustainable.

4. Conclusions

Reflectins are insoluble structural proteins with high biotechnological potential since can be processed into bio-based materials with variable architecture (films, fibers, diffracting grating) and rare electro-optical properties. However, high amounts of protein are required for materials production. Therefore, the overall goal of this study was to provide a robust, productive, easily scalable, and less expensive method for the purification of reflectin proteins. To test the robustness, we used two proteins (R1b and R6) that are naturally found in different cephalopod species and animal tissue and differ in length and sequence composition. Among the three purification methods tested, the washing of the IBs with low-concentrated denaturing buffers showed to be the most promising, allowing high purification yield, low protein loss and good purity in less amount of time.

This report focuses on a distinct strategy for reflectins purification, as we applied IB washing without a prior chromatographic method. Contrary to IMAC and reverse-phase chromatography the developed method was not limited by the presence and the binding capacity of the stationary phase.

Despite the need for adaptations on a case-to-case basis, we believe that this work can inspire other researchers working with structural proteins to apply alternative non-chromatographic methods for the downstream processing steps.

CRediT authorship contribution statement

Iana Lychko: Methodology, Investigation, Validation, Visualization, Writing – original draft. **Cátia Lopes Soares:** Methodology, Investigation, Validation, Writing – original draft. **Ana Margarida Gonçalves Carvalho Dias:** Methodology, Supervision, Writing – review & editing. **Ana Cecília Afonso Roque:** Methodology, Resources, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2023.123736.

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