In vitro investigation of the photoprotection mechanism of Light Harvesting Complex II

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CHAPTER 4

Production of ^{13}C Lutein rLhcb1 for NMR studies

ABSTRACT

In this chapter, we employed recombinant expression to design a selectively labelled LHCII pigment-protein complex with only lutein ^{13}C isotope-labelled among all the pigments participating to the protein refolding. We overexpressed in *E. coli* to obtain high amounts of the apoprotein Lhcb1 from *Arabidopsis thaliana*, which is one of the most abundant polypeptides of the Light-Harvesting Complex LHCII.

To achieve selective lutein labelling, the unlabelled purified Lhcb1 apoprotein from *E. coli* was refolded in the presence of pigment mixture which consisted of unlabelled Chl *a* and *b*, violaxanthin and neoxanthin and uniformly ^{13}C-labelled lutein. Unlabelled Chlorophylls and carotenoid pigment mixtures were extracted from spinach leaves and the carotenoids were further purified via HPLC, while ^{13}C lutein was obtained from biosynthetically ^{13}C enriched *C. reinhardtii* cells by total pigment extraction and purification via HPLC. The labelling efficiency of the recombinant protein was determined by mass spectrometry (MS) and estimated to be ~38%. The reconstituted ^{13}C lutein-rLhcb1 complexes were purified using Ni^{2+}-column purification and sucrose gradients and subsequently analysed by UV-Vis, CD spectroscopy and size-exclusion chromatography (SEC). These characterizations confirmed that the recombinant protein is well folded and in monomeric form.

INTRODUCTION

In th previous chapters the attention was focused on the native trimeric form of LHCII from *Spinacia oleracea*, here the focus is shifted towards a recombinant system to study the characteristics of selective carotenoids in LHCII. In this chapter, we describe an approach that combines biosynthetic isotope labelling and pigment purification from green algae, recombinant expression, and pigment reconstitution to obtain ^{13}C lutein-rLhcb1 for characterization by NMR spectroscopy. An important advantage of using recombinant protein, as alternative to native ones, is the possibility to label one specific component of the protein-pigment complex that otherwise could not have been possible with the standard growing procedure. In this case, we aimed to have unlabelled protein and among all pigments components only the lutein to be ^{13}C labelled.

Recombinant LHCs have been a great tool to study the protein function. Site-selective mutagenesis has been used to block specific pigment binding sites of interest to understand how the relative pigments were interacting, and how their presence could affect the folding [1,2]. It has been investigated the selectivity of the binding pockets towards specific pigments varying the compositions of the pigments mixture, without site-selective mutagenesis [3].

The recombinant LHCII so produced has the characteristic to have the backbone of the protein and all the cofactors non-isotopically labelled ad exception of only one, the lutein. The steps involved in the protocol of the recombinant proteins are pretty straightforward. The gene is cloned in the vector, transformed in the host and induced in the culture. At this point, the protein overexpressed is ready to be purified and characterized. Over the past twenty decades, many attentions were given to the importance of this technique which is widely documented in many reviews [1-9]. For most recombinant expression studies, *E. coli* is used as a host organism due to the fast growth. The duplication time is 20 minutes, which means that in a few hours the host is ready to be induced, although; in some occasion; it has been noticed that the expression of the recombinant protein might cause a sensible decrease of the duplication time [10]. When the foreign gene is introduced in the new host it will be expressed in a different environment regarding pH, osmolarity and redox potential which might interfere. In addition to it, the expressed protein with low or null solubility in water leads to the formations of build-ups of protein aggregated named inclusion bodies (IBs) [11]. In some case, therefore, the formation of IBs can be an advantage especially when the protein can be easily refolded *in vitro*.

The apoprotein Lhcb1 once purified from the *E. coli* cells does not have the proper folding because the host organism is unable to produce the cofactors essential to transform the apoprotein in the final pigment-protein complex. The Lhcb1, therefore, is folded in presence of a pool of cofactors such as Chls, carotenoids, and lipids, which are arranged accordingly to the protein backbone.

LARGE SCALE OVEREXPRESSION AND PURIFICATION OF Lhcb1 APOPROTEIN IN E. coli

OVEREXPRESSION

Lhcb1 apoprotein was overexpressed in *E. coli* BL21 pLysS. Tests were performed at different temperatures, 18°C and 37°C, in a range of time between 3 hours and overnight. As shown in Figure 4.1 the vector at 18°C overnight does not give any overexpression after induction of the cells with IPTG, while the same vector at 37°C, after 3 hours from induction, shows overexpression of 10 mg starting from 200 ml of culture.

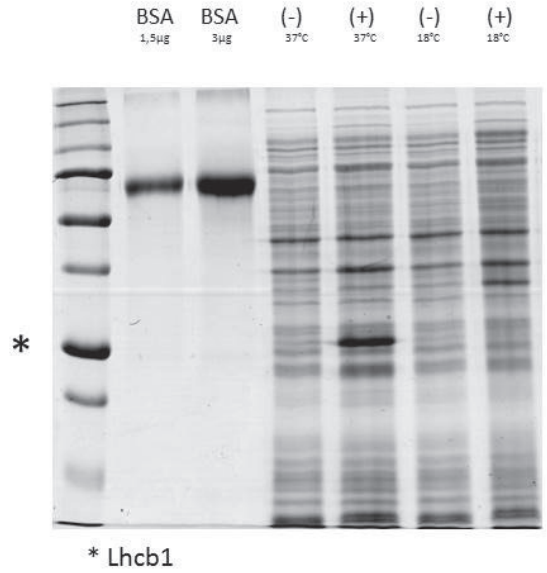

Figure 4.1 SDS page of overexpressed Lhcb1 in BL21 pLysS. From left to right: ladder, BSA 1.5 ug, BSA 3ug, 37°C (-) IPTG, 37°C 3hr (+) IPTG, 18°C (-) IPTG, 18°C O/N (+) IPTG

To improve the overproduction yield, two more strains were tested. These two strains, BL21-CodonPlus and BL21-RP*, showed an increase in the yield because of better compatibility between their tRNA and the one of the apoprotein. Therefore we decided to put aside the first tested strain, BL21-pLys, due to codon bias effect [12]. In Figure 4.2 a denaturing SDS gel is shown with the tested conditions. As for the previous strain, the strains were tested using different growing conditions from 3 hours after induction until overnight. The best yield was given by the strain BL21-codonPlus with the optimum conditions of 37°C and 3 hours growing time from induction. Overnight growing was disregarded to avoid protein degradation, which is more likely to happen after a long time from the induction due to *E. coli* stress [13,14]. For the BL21-CodonPlus at 37°C after 3 hours from induction, the estimated yield was about 30 mg of protein from 200 ml of cells culture. The final estimation was done after the purification of inclusion bodies from the cells.

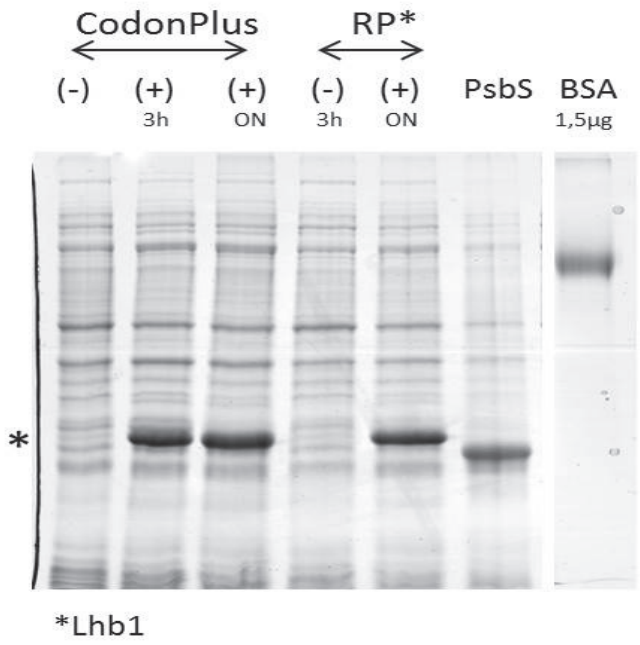

Figure 4.2 SDS page of overexpressed Lhcb1-pExp5 in BL21-codonPlus and BL21-RP. From left to right, CodonPlus 37°C (-) ITPG, CodonPlus 37°C 3h, (+) ITPG, CodonPlus 37°C O/N, (+) ITPG, RP* 37°C (-) ITPG, RP* 37°C O/N (+) ITPG, control PsbS at 22 kDa, BSA 1.5 ug*

Under equal conditions, both strains are suitable for the protein growth giving a similar yield. The strain CodonPlus was chosen, over RP*, for the overexpression of Lhcb1.

The final overexpression of the apoprotein, Lhcb1 gene from *Arabidopsis thaliana*, was performed in 500 ml of *E. coli* BL21-CodonPlus, using 1 L Erlenmeyer flask, of preheated at 37°C, LB medium [15] with antibiotics, ampicillin and chloramphenicol, inoculating with ~300 µl of pre-culture, from -80°C glycerol stock (OD_{600}~ 0.6). The culture was grown at 37°C in flasks while stirring with a speed of 220 rpm until the OD_{600} reached 0.6. At this stage, the cells were supplied with IPTG and kept growing until the OD_{600} was around 3, which corresponds to 3-4 hours after induction. All cells were pelleted down and stored at -80°C until the next step of purification. The overexpression yield was in a range between 150 and 200 mg per 1 L of culture.

With the intention of increasing the overexpression yield, *E. coli* cells were grown using a 2 L bioreactor (Schott-Buran). The bioreactor allows a better overproduction due to change in the growing conditions. In particular, with the set-up we are equipped with, via the use of an external pump, air was constantly supplied to the culture and the internal culture temperature was monitored with a thermometer in contact with the culture. Higher culture oxygenation allows for a longer growing time because the stationary phase is reached later than in normal conditions [16,17]. Because the set-up was not provided with a channel for feeding the culture, the maximum growing tested time was up to overnight after the induction. Similarly, for the growth of bacteria in the flask, cells were harvested after 3-4 hours of growing from induction in order to prevent any food stress which will have a negative impact in the overall of the protein production. In particular, food stress is responsible for the arrest of all metabolic activity and growth. This leads to the production of new enzymes, such as protease, lipases, and substrate capturing [18-

20]. Under these optimized conditions, the overproduction yield was around 300 mg per 1 L of culture.

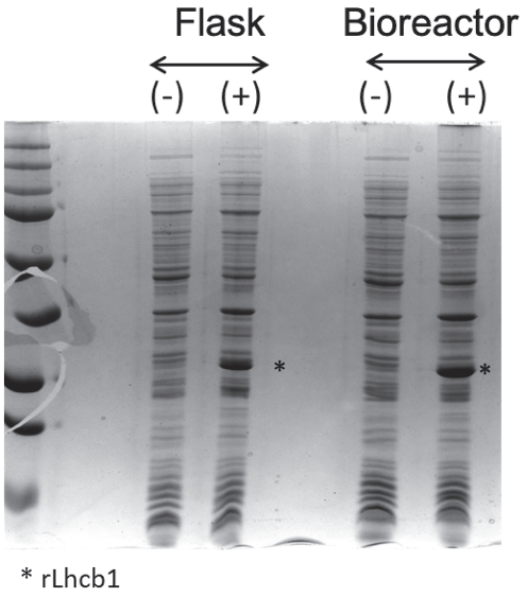

Figure 4.3 SDS polyacrylamide gel of rLhcb1 overexpression at 37°C. From left to right, Marker, flask (–) IPTG, flask (+) IPTG, bioreactor (-) IPTG, bioreactor (+) IPTG

PURIFICATION

As already mentioned, eukaryotic membrane proteins are often accumulating in cells in the form of protein adducts called inclusion bodies (IBs) [21].
The IBs, hosting Lhcb1 were purified from pelleted cells accordingly to the protocol of Krishnan *et al.* [22,23]. Several washing steps using Triton buffer (Tris 20 mM, Triton x-100 0,5 % w/v, ß- Mercapto-ethanol 1 mM) were performed in order to solubilize most of the impurity as showed in Figure 4.4. The pelleted cells have been solubilized in lysis buffer (Tris 50 mM. sucrose 2.5% w/v, EDTA 1 mM, Lysozym 10 mg/ml pH 8). The total sample (T) was taken after have added detergent buffer (NaCl 20 mM, Deoxycholic acids 1% w/v, Tris 20 mM, EDTA 2 mM, ß-Mercapto-ethanol 10 mM, pH 7.5). The solution was spun down and separated from the supernatant (S_n) while the pellets were dissolved in fresh Triton buffer (P_n).

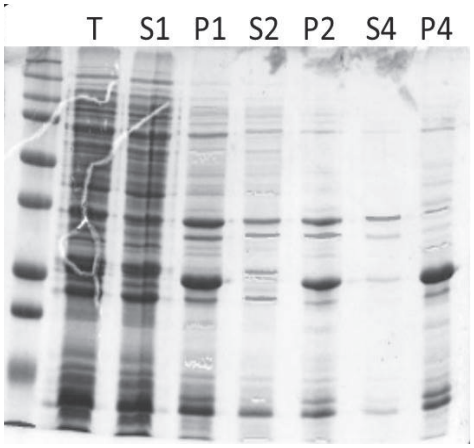

Figure 4.4 SDS page of purified Lhcb1 from left to right: Marker, total protein, S_1, P_1, S_2, P_2, S_4, P_4.

Following 3 washing steps, the apoproteins pelleted in inclusion bodies was solubilized in TE buffer (Tris 50 mM, EDTA 1 mM, pH 8, ß-Mercapto-ethanol) and stored at -20°C (P_4) until refolding. From a total amount of 125 mg of protein in cell pellet, 90 mg of total protein in inclusion bodies was purified.

^{13}C LABELLING OF CHLAMYDOMONAS REINHARDTII

C. reinhardtii cells, (strain JVD-1B[pGG1]) were grown in our laboratory using a home-built growth chamber. Algae were grown under constant light intensity consisting of eight individual cool white LEDs that gave approximately 50 µmol/m^{2}s of light in the photosynthetically active region equipped with a LED outputs of 3 watts and a temperature colour of 6500K [24]. The growth temperature was set at 25°C ± 0.2 °C, in Tris-acetate-phosphate medium at pH 7.0 (TAP medium) [25] as food source. *C. reinhardtii* algae were isotope labelled using acetic acid_1,2-$^{13}C_2$ (99% ^{13}C, Cambridge Isotope Laboratories, Inc.) replacing the acetic acids as component of the TAP medium. Green algae are classified as a mycotrophic system, which means that they are able to use several carbon sources. In this case, the available carbon sources were both the ^{13}C labelled acetic acid from the medium and ^{12}C from CO_2 coming from the air that is naturally dissolved in the medium and that will be taken up during the respiration process. The availability of both carbon sources reduces the ^{13}C label efficiency because ^{12}C will also be present [26].

The algae were grown starting from a plate culture, and then with the use of a sterile inoculating loop, a single colony was harvested and diluted in a 5 ml volume of TAP medium. From the stock culture, 1 ml (with OD_{750} of 1) was used to inoculate 500 ml of ^{13}C labelled TAP medium. After approximately 4-5 days, with the optical density equal to 2-2,5 at 750 nm, the culture was ready to be harvested.

PIGMENT EXTRACTION

Unlabelled pigments were extracted from fresh market spinach leaves.
In the total pigments extraction both Chls and carotenoids are presents while with the carotenoids extraction these are separated from the Chls. Labelled pigments were extracted from ^{13}C isotopic labelled *C. reinharditii* [27].
In short, chloroplasts were extracted from homogenized spinach leaves and washed with wash buffer (Sorbitol 50 mM, Tricine 5 mM and EDTA 1 mM with pH 8) which allowed to purify the thylakoid membrane. Pigments were subsequently extracted from the pelleted thylakoid using acetone buffered with di-sodium carbonate. The cellular components were taken out from the dissolved pigment in acetone via centrifugation. Dissolved pigments were then extracted using diethyl ether which was evaporated using a rotary evaporator. After extraction, pigments were quantified [28], dried and stored at -80° under a nitrogen stream until further use. For the carotenoids extraction, the procedure was almost the same with the exception of the carotenoids saponification performed overnight by potassium hydroxide. After this treatment, carotenoids were separated from Chls because do the latter are not soluble in diethyl ether but rather in the water phase. Carotenoids content in the mixture was determined using the respective extinction coefficients [29].

HPLC ISOLATION

Individual pigments were purified from the -80°C stock of total Chls and carotenoids using high-pressure liquid chromatography (HPLC). The sample was dissolved in acetone and spun down prior to column injection (Gemini 5 um NX-C18 110, LC column 250 x 10 mm), to prevent any pigments aggregates or salts (NaCl or $NaSO_4$ used during the pigments extraction) from going into the column and occlude it. The separation was performed using an Agilent technical 1200 connected in series with an Agilent technical quadrupole LC/MS 6130, protected with a guard column 10 x10 mm. The protocol used refers to what Gilmore *et al.* [30] described in their paper with some adjustments. The pigments were eluted using a gradient between 2 different eluent mixture, A and B. Eluent A is composed of 70% acetonitrile, 20% methanol, and 10% water while eluent B contained 32% ethyl acetate and 68% methanol. A flow rate of 5 ml/min was used. One purification run from the total pigment extraction took 48 minutes with a gradient of 0-80%. Additional 6 minutes were used to clean the column before the next injection. During the purification, from the total mixture of pigments, four main elution peaks were identified combining the information from MS and UV-Vis spectroscopy.

	Time (minutes)	%A	%B
1	0	100	0
2	2	20	80
3	47	20	80
4	48	0	100
5	54	0	100

Table 4.1 Scheme of gradient elution going from 100% A at time zero to 100% B in 54 minutes.

The first peak with the mass of 600 g/mol, is in agreement with the mass of violaxanthin and neoxanthin. Lutein is the second peak with a mass of 568,871 g/mol. The third peak with a mass of 900 g/mol, is Chl *b,* and the last peak with a mass of 893 g/mol, is Chl *a*.

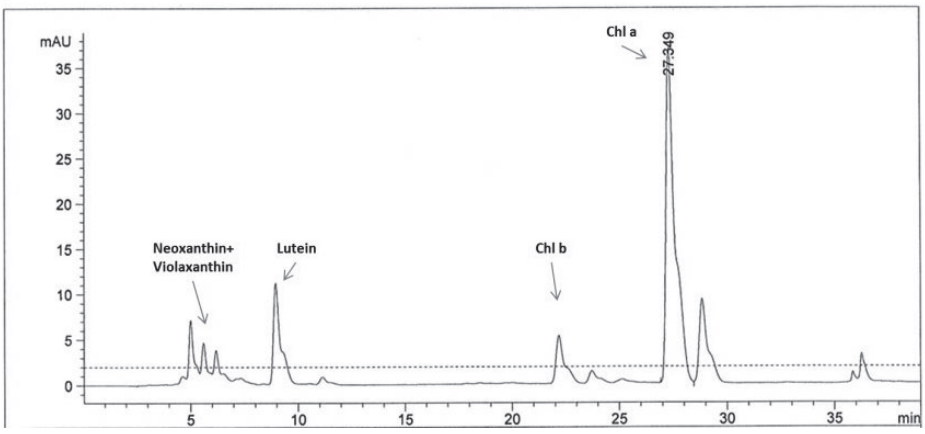

Figure 4.5 HPLC elution profile of pigment isolation from total pigments extracted from spinach. In the y-axis is report the absorption intensity measured at 440 nm.

During the run, a small aliquot of the sample was collected and dissolved in 50% acetonitrile/water solution. The sample was injected in LC/MS using as eluent 1% of acetic acid with a flow rate of 0,5 ml/min. Pigments are not only light sensitive but also oxygen sensitive. In fact, the presence of oxygen activates processes as epoxidation or bleaching [31] and the MS analysis, of the eluted aliquot, was a great tool to discriminate oxidized pigments from the non-oxidized. The two species of the same compound had different elution times, therefore multiple bands were observed which means different MS spectra and absorption profiles. Pure fractions were dried under a stream of nitrogen and stored at -80°C until further use. Chlorophylls content was quantified accordingly to Porra *et al.* [28], while the carotenoid content was determinate accordingly to [29].

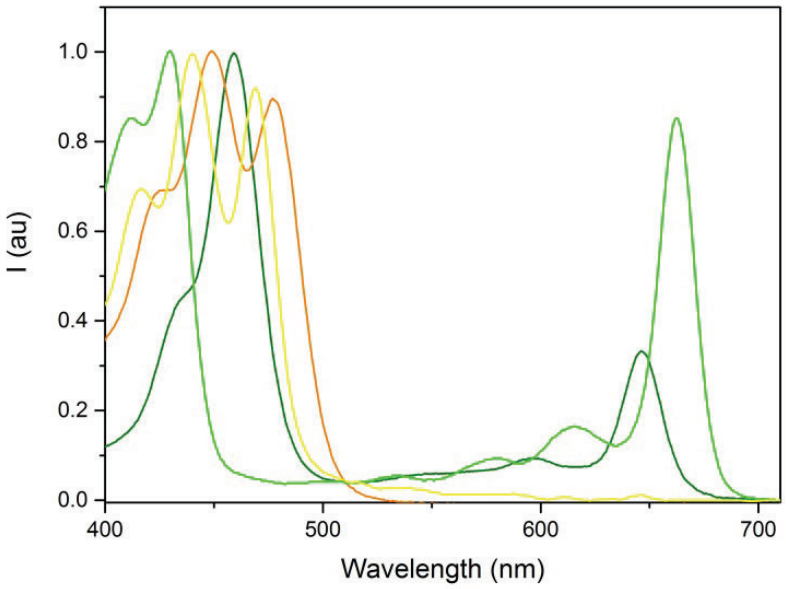

Figure 4.6 UV-Vis absorbance spectra of isolated pigments, normalized for the maxima of absorption. Chl a in light green, Chl b in dark green, lutein in orange and Vio+ Neo in yellow.

^{13}C QUANTIFICATION

A small aliquot of the eluted fractions was analysed via MS to get their mass profile. This was an essential tool not only to identify the pigment types but also to determine the ^{13}C labelling profile. In this context, we concentrate our attention on the lutein sample. Before to proceed with the analysis of the mass spectra of ^{13}C lutein, we had to verify that the mass visible in the spectra is the real mass minus one water molecule, which has a mass of 18,01 g/mol. This means that in case of ^{12}C Lutein, of which the molecular weight is 568,871 g/mol, the expected mass is around 550,86 g/mol, to which corresponds a peak in the mass spectra profile with a relative intensity percentage of 100. A small percentage of the compound with the real mass around 568.1 g/mol is also visible as shown in Figure 4.7.

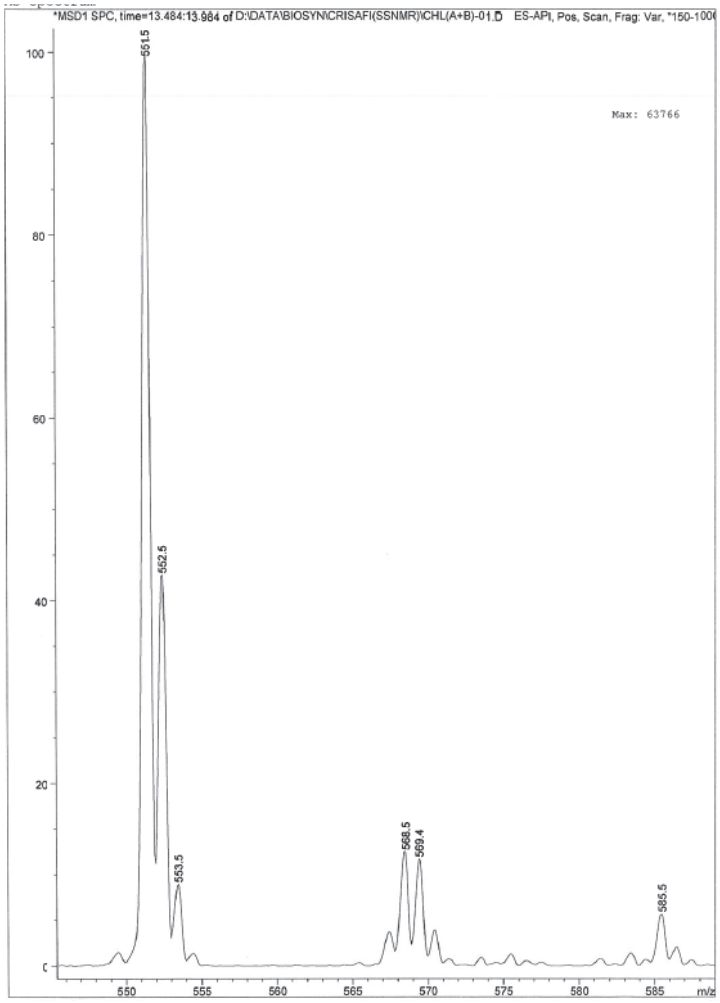

Figure 4.7 MS profile of na lutein.

Very much different is the profile of the labelled lutein. Ideally, for a molecule that is synthesized, we would have expected an increase in the total mass equal to 1 x C_n, corresponding to about 100% of ^{13}C labelling. In the case of lutein, from the chemical formula, $C_{40}H_{52}O_2$, there are forty atoms of carbon which means an increase equal to 40 g/mol, for a final mass of 608,871 g/mol. From a simulation of the expected profile of the compound, we would have expected only one peak with mass of 590,86 g/mol (608,871-18,01 g/mol).

Algae were grown under constant light, which increased the probability of fixating the CO_2 from air [32].

The proof that the algae not only take up carbons from ^{13}C acetic acid in the medium but also fixate carbons from CO_2 is shown in the MS spectra of ^{13}C lutein, as reported in Figure 4.8.

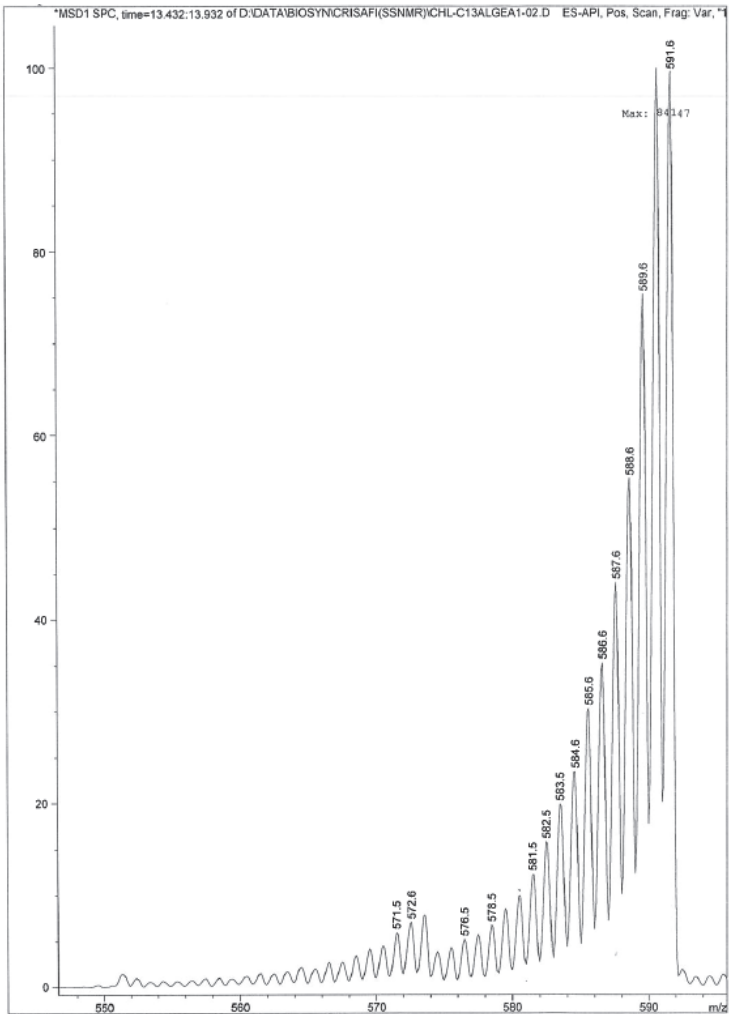

Figure 4.8 MS profile of ^{13}C lutein.

It is fascinating to observe the mass distribution profile of the ^{13}C lutein. The peaks distributions starting from the fully ^{13}C labelled compound toward the natural abundance are observed in Figure 4.8 going from right to left. Two peaks with equal relative intensity, of 100, are visible and correspond to 40 and 39 carbon atoms labelled and left from these there is a series of peaks, precisely 38 more, which correspond to molecules with one less ^{13}C labelled carbon each, until the very last peak which has 40 ^{12}C carbons. To estimate the percentage of labelling, the ^{13}C abundance of the different peaks with the occurrence of more than 10 in intensity was taken into account. Below this threshold, the values of peaks with less than 29 ^{13}C carbons were neglected. From the mass spectra profile shown in Figure 4.8 the total intensity of the peak fractions above the threshold is indicated by the formula:

$$\text{Total intensity} = \sum_{i>18}^{n} I_n$$

Giving a total intensity of 518.
The contribution of each fraction above the threshold was calculated according to the formula reported below

Relative Intensity= I_n / $\sum_{i>18}^{n} I_n$

In conclusion, the $^{13}C_{40}$ and $^{13}C_{39}$ lutein molecules together account for 38,61% of the total peak fractions. After that we have 14,5% of $^{13}C_{38}$,10,61% of $^{13}C_{37}$, 8,5% of $^{13}C_{36}$ and so on.

REFOLDING AND CHARACTERIZATION OF ^{13}C LUTEIN rLhcb1

REFOLDING OF Lhcb1USING WHOLE-PIGMENT EXTRACT

Refolding of recombinant Lhcb1 was first tried as the protocol reported by Natali *et al.* [27]. Briefly, ~800 µg of protein in inclusion bodies was pelleted and solubilized in 400 µl of TE buffer (Tris 20 mM, EDTA 1 mM, HEPES 200 mM, sucrose 5% w/v) with 400 µl of recombinant buffer (Sucrose 5% w/v, LDS 4% w/v, HEPES 200 mM, pH 7.5). The protein was denatured at 98°C in 1 minute. While the protein solution was cooling down, pigments were prepared, 500 ug of Chl (*a*+*b*) and 80 µg of carotenoids. The new detergent, OG, was added with the final concentration of 2% and kept on ice for 10 minutes. In order to precipitate the LDS present from the inclusion bodies purification, KCl was added, with a final concentration of 200 mM and the sample was kept on ice for 20 minutes before it was pelleted at 15800 x g for 10 minutes at 4°C. After that, the refolded protein was separated from unbound pigments using Ni^{2+} column and by using linear sucrose gradients. In order to minimize protein losses by skipping the Ni^{2+} column step, also a variation on the protocol was tried using Lhcb1 apoprotein that was first purified with urea wash, as Krishnan *et al.* [22] optimized for PsbS. In this case, the protein was purified from the inclusion bodies with the use of urea buffer (100 mM sodium phosphate, Tris 100 mM, Urea 8M, LDS 0,05% pH 8).

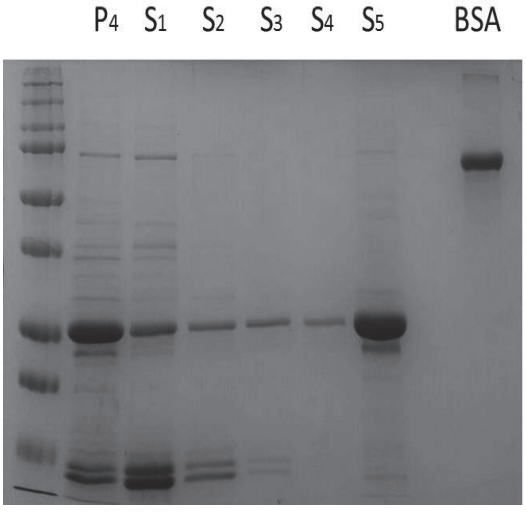

Figure 4.9 SDS page of urea wash purification of Lhcb1 from left to right: Marker, P_4, S_1, S_2, S_4, S_5 and BSA ~3µg

With the help of the urea, all the impurity will be washed away in the supernatant (S_1-S_4) while the apoprotein will be present in the pellet. The Lhcb1 apoprotein was solubilized in buffer (sodium phosphate 100 mM, Tris 100 mM, Urea 8M, pH 8) with high amount of LDS (0,5%). After the overnight incubation, the apoprotein was fully solubilized (S_5), and the buffer was exchanged to TE buffer, the same one used for the protein refolding, using a PD10 column. Figure 4.9 shows that until wash S_4 there is some inevitable loss of the Lhcb1, however, the apoprotein is recovered in S_5.

REFOLDING OF Lhcb1 USING MIXTURE OF HPLC-PURIFIED PIGMENTS

In a first trial, the LHCII pigment mixture for reconstitution was created by mixing stoichiometric amounts of HPLC purified Cars and Chl pigments. I compared, pigment-refolded, rLhcb1 samples that had the same apoprotein and were differing only for the pigment mixtures used for reconstitution. The "rLhcb1 standard" was prepared as in the published protocol [27], using total pigment extraction from spinach and "rLhcb1 mix pigments" was prepared using a mixture of HPLC purified pigments in stoichiometric ratios. As shown in Figure 4.10a, the absorption and CD profiles of the "rLhcb1 mix pigments" sample is not in agreement with the profile of "Lhcb1 standard" obtained using the standard protocol and indicates incomplete pigment reconstitution.

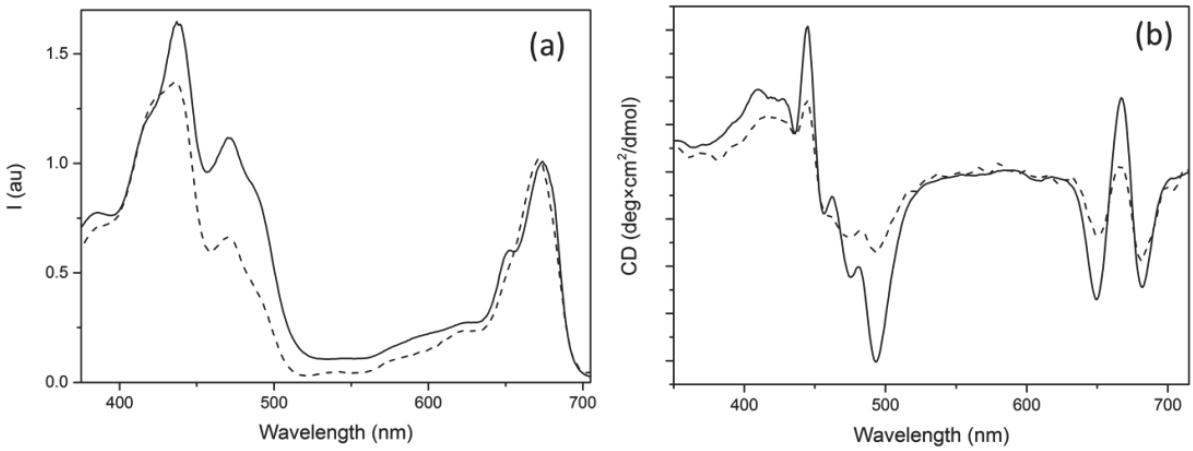

Figure 4.10 (a) UV-Vis spectra normalized for the peak at 674 nm. Dash is the rLhcb1 refolded with HPLC purified pigments, solid line is rLhcb1 with a mixture of extracted pigments. (b) CD spectra normalized for the concentration. Solid line is the rLhcb1 refolded with whole pigments extractions, dash line is rLhcb1 with the mixture of HPLC purified pigments

The refolded Lhcb1 with the HPLC-purified pigment mixture is lacking in Chl *b*. This result might be explained by the absence of lipids that are extracted together with the pigments in total-pigment mixtures used to reconstitute the Lhcb1 standard, but that was lost during the HPLC purification. To our knowledge, previous studies were performed using pigment mixtures extracted from thylakoids [23] or the apoprotein was reconstituted in presence of lipids in order to have the folded pigment-protein sample in liposomes [33,34] and lipids might be essential for correct folding and pigment binding *in*

vitro. Proteins in native environments are folded in presence of lipids, of which phosphatidylglycerol is also essential for trimer formation. Also, membrane proteins are, per definition, more stable in the presence of lipids than in a detergent environment, which might explain as well why refolding the protein in a mixture without lipids does not lead to a correct folding.

REFOLDING OF Lhcb1 USING Chl WHOLE-PIGMENT EXTRACTS AND HPLC-PURIFIED CAROTENOIDS

The pigment mixture for the reconstitution was changed in consequence of the previous results. The mixture consisted of Chls from a whole-pigments extraction, while the Cars mixture mimicked the native ratio between Neo, Vio, Lut using HPLC purified Cars.
In this way, from the whole pigments extraction, eventual co-extracted lipids would be available to allow proper refolding while the carotenoids mixture was made combining the HPLC purified pigments of interest. Excess of Cars, especially ^{13}C lutein, should promote a preferential binding of the ^{13}C lutein to the lutein L1 and L2 binding pockets [35,36]. CD and UV-Vis absorption confirmed the success of the refolding and the protocol was up-scaled for the need of producing milligrams of ^{13}C lutein-rLhcb1 for study by NMR. In particular, for each preparation 4.8 mg of protein in inclusion bodies were refolded as in the protocol in presence of 3 mg of chlorophylls, from the whole pigments extraction, and 480 µg of HPLC purified carotenoids of which half is ^{13}C Lutein. After the Ni^{2+} purification, the sample was purified via sucrose gradient.

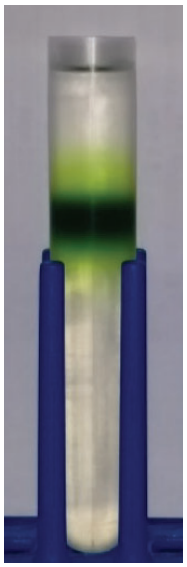

Figure 4.11 Sucrose gradient with 0.06% βDM, 0.01 M HEPES, pH 7.6 of rLhcb1 after purification with Ni^{2+} column.

Aliquots from the fraction were loaded on an SDS denaturing gel page to verify the presence of the protein as showed in Figure 4.12 below.

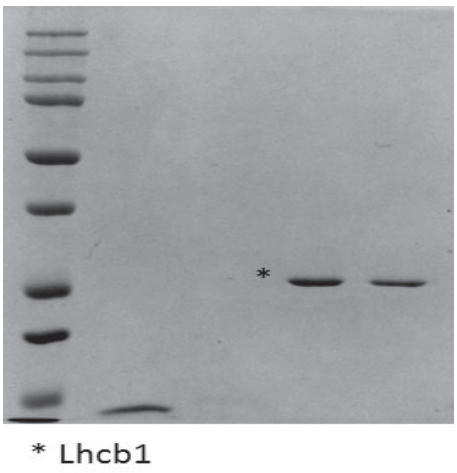

Figure 4.12 Denaturing SDS gel page of rLhcb1. From left to right Ladder, Wash I, Flow through, Fraction II and Fraction I.

From the SDS gel page analysis, in both fractions, I and II, the presence of the Lhcb1 protein is verified which has a band around 25 kDa. Figures 4.13 and 4.14 show the UV-Vis absorption and CD profiles of pooled fractions of ^{13}C-lutein rLhcb1, confirming that the pigment-protein complexes were properly folded.

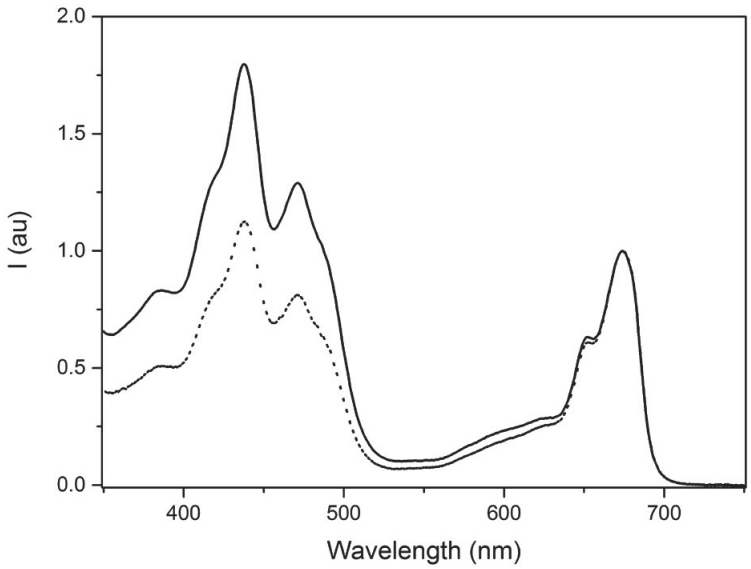

Figure 4.13 UV-Vis spectra of the final sample of ^{13}C lutein-rLhcb1. The spectra are normalized for the peak at 674 nm, Fraction I in the solid line and Fraction II in the dotted line.

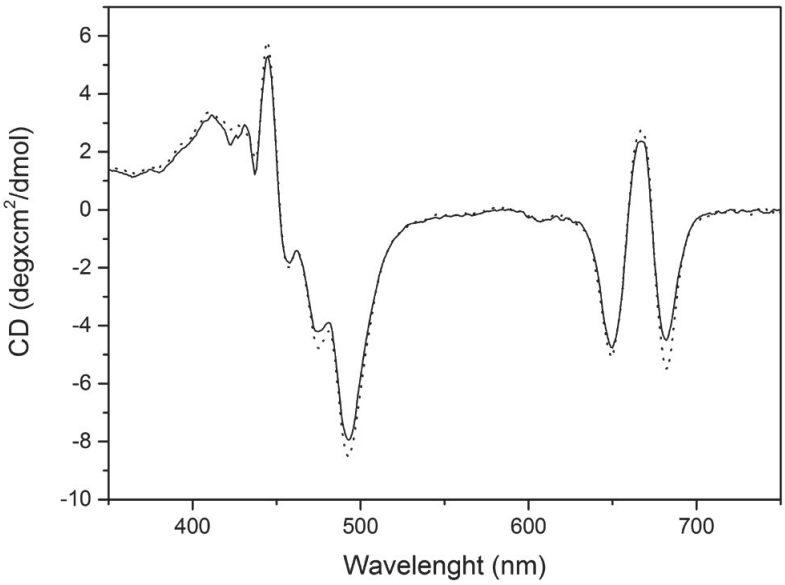

Figure 4.14 CD spectra normalized for the concentration of recombinant ^{13}C lutein -rLhcb1. Fraction I is the solid line and fraction II is the dotted line.

The CD spectrum, Figure 4.14, is in agreement with literature data for recombinant, monomeric LHCII which has a characteristic profile in comparison to the trimeric state [33]. The monomeric oligomer state is further confirmed from size exclusion chromatography [33,37].

The elution profile of rLhcb1 was compared with the one from LHCII monomers that were extracted from spinach leaves, collecting the monomer band from a sucrose gradient. From the elution profile in Figure 4.15, we can conclude that indeed the rLhcb1 is present as a monomer. The elution profile of rLhcb1 was compared with the profile from a mixture of gel-filtration protein standards (data not showed) with molecular weights in the range between 670 kDa and 1350 Da (Bio-rad) [38], estimating a monomer size around 90 kDa for the monomer pigment-protein complex embedded in detergent micelles.

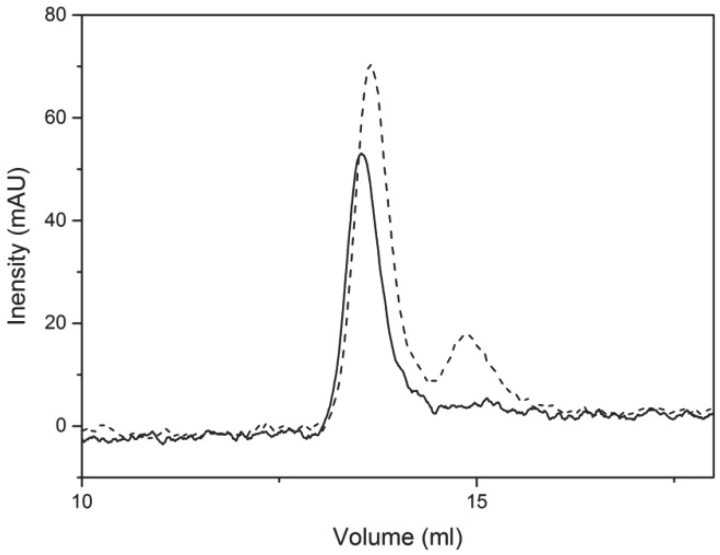

Figure 4.15 SEC chromatogram at 280 nm of rLhcb1 from A. *thaliana in solid line compared with native monomeric LHCII extracted from spinach in dashed line.*

Pigments composition was checked using HPLC on reverse phase C18 analytical column (Phenomenex) using a protocol based on Gilmore *et al.* [39]. The HPLC chromatogram in Figure 4.16 was compared with the pigment profile of native LHCII trimer from spinach, as the standard. We can conclude that all the pigments got inserted with the observation of a slight difference in the insertion of Lut and Vio. The amount of Vio inserted is less but this is acceptable because the pigment for monomer is loosely bounded at the periphery of the pigment-complex which likely can be lost in the insertion/purification process, in addition, this site does not have selective affinity, while Neo binding site is 100% selective for this pigments with interacts of Chl *b* [1,40]. From the chromatogram it seems that more lutein got inserted occupying other binding sites maybe, the Vio binding site was more likely occupied by the Lut rather than from the Vio [1,40].
The amount of pigments inserted, see Table 4.2, was estimated from the HPLC. Chl (*a*/*b*) is equal to 1.38 which is close to the theoretical of 1.33 The estimation was compared with the literature of native LHCII, in which it has been reported a value for Chl (*a*/*b*) around 1.33 for higher plants [41,42].

	Neo	**Vio**	**Lut**	**Chl b**	**Chl a**
rLhcb1	0.8 ±0.04	0.3 ±0.015	2.3 ±0.115	5.8 ±0.29	8 ±0.4

Table 4.2 In the table amount of pigment estimated from HPLC measurements. With the assumption that the Chl a content of 8 molecules.

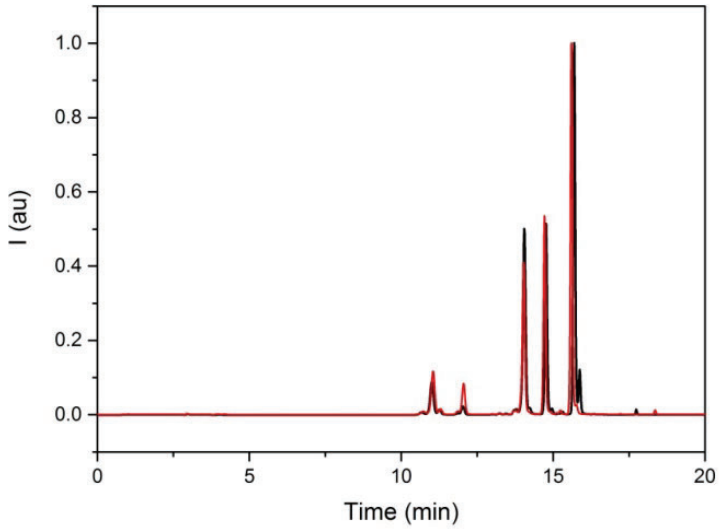

Figure 4.16 HPLC Chromatogram, with a detection wavelength of 440 nm, of rLhcb1 black line and native LHCII trimer from spinach leaves in red line. Spectra were normalized for the Chl a peak.

The collected bands of different fractions were flash-frozen and stored at -80°C in the presence of sucrose as a cryo-protectant, until use.

CONCLUSIONS

We can conclude that the recombinant and reconstituted ^{13}C lutein rLhcb1 was correctly refolded and the upscaling of the production was successful to yield 12 milligrams of protein to be further study with solid-state NMR spectroscopy.

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