An improved method for estimating R-phycoerythrin and R-phycocyanin contents from crude aqueous extracts of *Porphyra* (Bangiales, Rhodophyta)

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Received: 31 January 2006 / Revised and Accepted: 13 June 2006 / Published online: 29 November 2006
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Abstract One frequently-cited method for determining phycoerythrin (PE) and phycocyanin (PC) contents from crude aqueous extracts of red seaweeds utilizes peaks and troughs of absorbance spectra. The trough absorbance values are used to establish a linear or logarithmic baseline attributable to background scatter of particulate cellular debris not removed by centrifugation. Pigment contents are calculated by subtracting baseline values from PE and PC absorbance peaks. The baseline correction is intended to make the method independent of centrifugation time and/or speed. However, when crude extracts of *Porphyra* were analyzed using this protocol, R-PE and R-PC estimates were significantly affected by centrifugation time, suggesting that the method was not reliable for the genus. The present study has shown that with sufficient centrifugation, background scatter in *Porphyra* extracts can be removed, the remaining spectrum representing the overlapping absorbance peaks of water-soluble pigments in the

extract. Using fourth derivative analysis of *Porphyra* extract absorbance spectra, peaks corresponding to chlorophyll, R-PE, R-PC, and allophycocyanin (APC) were identified. Dilute solutions of purified R-PE, R-PC and chlorophyll were scanned separately to identify spectral overlaps and develop new equations for phycobilin quantification. The new equations were used to estimate R-PE and R-PC contents of *Porphyra* extracts and purified R-PE, R-PC and chlorophyll solutions were mixed according to concentrations corresponding to the sample estimates. Absorbances and fourth derivative spectra of the sample extract and purified pigment mixtures were compared and found to coincide. The newly derived equations are more accurate for determining R-PE and R-PC of *Porphyra* than previously published methods.

Key words fourth derivatives · phycobilins · pigments · red seaweeds · spectroscopy

Abbreviations

APC Allophycocyanin
Chl *a* Chlorophyll *a*
Chl *d* Chlorophyll *d*
R-PC R-phycocyanin
R-PE R-phycoerythrin

Introduction

In red algal ecological, physiological and aquaculture studies, there is a need for a quick, easy and reliable

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method for determining phycoerythrin (PE) and phycocyanin (PC) content. One of the most frequently cited phycobilin quantification techniques (Beer and Eshel 1985) utilizes the spectral properties of crude aqueous extracts to estimate the pigment contents of sample tissues. The samples are ground, extracted in phosphate buffer, centrifuged and the absorbance values of the supernatant corresponding to peaks and troughs of PE and PC are recorded. The equations developed by Beer and Eshel (1985) were designed to correct for the scatter and absorbance of cellular/particulate debris within the crude extract that had not been removed by centrifugation and were thus relatively insensitive to centrifugation time and speed treatments.

Theoretically, an absorbance scan of a crude aqueous extract following centrifugation should contain the absorbance spectra of the pigments dissolved in the extract plus the scatter and absorbance of particulate debris. Beer and Eshel (1985) reported that the presence of suspended particulate debris within an extract would account for a more or less linear sloping baseline (Figure 1). Absorbance of PE and PC could thus be determined by subtracting the baseline from the total absorbance of the PE and PC absorbance peaks. If the extract were to be centrifuged more (or less), the slope of the linear baseline would change, but the distance between the baseline and the pigment absorbance peaks would remain the same (Figure 1). Therefore, multiplying the calculated absorbance of

Figure 1 Theoretical behavior of the absorbance scan and linear baseline according to the methodology developed by Beer and Eshel (1985). The dotted lines represent the behavior of the linear sloping baseline with increasing centrifugation as described by Beer and Eshel (1985).

PE and PC by their extinction coefficients (O'Carra 1965; Siegelman and Kycia 1978) should result in the same estimation of pigment content regardless of how much the samples are centrifuged. We found here that this was not the case for crude aqueous extracts of *Porphyra*. R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) contents calculated as per Beer and Eshel's (1985) recommended equations varied depending on the centrifugation time applied. Inconsistencies in the calculated phycobilin contents among centrifugation treatments shed doubt on the ability of Beer and Eshel's (1985) method to accurately determine R-PE and R-PC contents within *Porphyra*. Consequently, a detailed study on the effect of centrifugation on absorbance spectra of *Porphyra* crude extracts was performed. Specifically, the absorbance spectrum of the particulate debris (i.e., the baseline) was investigated separately from the absorbance spectrum of the pigments dissolved in phosphate buffer. The response of the baseline to increasing amounts of centrifugation is also discussed along with a detailed description of the individual and combined absorbance spectra of the dissolved pigments. Lastly, a new set of equations was developed that more accurately estimates the R-PE and R-PC contents of *Porphyra*.

Materials and methods

Samples of *Porphyra umbilicalis* Kützinger were collected from Wallis Sands Beach, Rye, NH (N43°07'8.6", W70°49'39.7") and *P. purpurea* (Roth) C. Agardh were collected from Hilton Park, Dover, NH (N43°01'40.4", W70°43'33.8"). On the day of collection, 2.0 cm discs were removed from the vegetative portion of *Porphyra* blades, blotted dry and weighed to the nearest milligram. Phycobilin extraction was optimized based on a preliminary comparison of grinding apparatus (mortar and pestle, glass homogenizers and microtubes with polypropylene pestles). Effectiveness of each apparatus was tested with and without liquid nitrogen and/or small quantities of sand. Extraction times in phosphate buffer ranging from 2 to 48 h were also compared. The following protocol represents the optimum combination of the above.

Samples consisting of two discs with a total weight of 75–120 mg were ground using a 145 mL porcelain

mortar and pestle (Coors 60316 and 60317) with approximately 30 mg sand. A total of 1.5 mL 0.1 M phosphate buffer [16.73% NaH_2PO_4 (anhydrous) and 83.27% $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$, pH 6.8] was used to rinse the ground tissue from the mortar into a 1.7 mL polypropylene microtube (scraping the ground tissue/buffer out with a small metal spatula) in three steps, each using 500 μL buffer. Following a 24 h extraction at 4°C, samples were centrifuged in a Fisher Scientific Marathon 16 km centrifuge at 17,000 g (maximum speed). Absorbance corresponding to measured peaks and troughs specific to *Porphyra* (455, 565, 592, 618, 645, and 730 nm) were recorded using a dual-beam UV-visible spectrophotometer (Helios Alpha) against a blank containing phosphate buffer.

The “absorbance” spectrum of the dissolved particulate debris (i.e., the baseline) was determined using two approaches: (1) monitoring changes in the absorbance spectrum of the supernatant following a series of centrifuge times at 17,000 g (3, 5, 10, 20, 30 and 60 min) and comparing changes to the absorbance spectrum of the pellet debris resuspended in fresh phosphate buffer; and (2) spinning the samples down completely until the spectrum remained unchanged, pouring off the supernatant and resuspending the pellet in clean phosphate buffer. The behavior of the particulate debris was monitored by analyzing the “absorbance” spectra of the resuspended pellets using the same centrifuge treatments described above.

Fourth derivative analysis (Smith and Alberte 1994; Butler and Hopkins 1970a, 1970b; Butler 1972) of absorbance scans from *Porphyra* extracts were performed to resolve the overlapping peaks of individual pigment spectra. Absorbance scans of solutions of purified R-PE, purified R-PC and chlorophyll were conducted separately and fourth derivative analysis applied to identify corresponding absorbance peaks within the *Porphyra* absorbance scans.

Purified R-PE (1 mg; Sigma P-8912) and purified R-PC (1 mg; Sigma P-7165) were mixed with 1 mL 0.1 M phosphate buffer (pH 6.8) to form concentrated solutions of each pigment. The R-PE and R-PC (mg mL^{-1}) contents in *Porphyra* extracts were determined using Beer and Eshel’s (1985) equation IV and the newly derived equations presented here. Diluted solutions of R-PE and R-PC were prepared using phosphate buffer according to pigment estimations. Diluted R-PE and R-PC solutions were combined into a single solution representative of each

Porphyra extract. Spectrophotometric analyses performed on the synthesized sample solutions were compared to the original *Porphyra* extract. Chlorophyll, extracted from *Ulva* sp. (Jensen 1978; Rowan 1989), was added to synthesized phycobilin mixtures to determine whether the absorbance spectrum of chlorophyll overlapped with the absorbance peaks of R-PE and/or R-PC.

Results and discussion

Characterizing the baseline

The equations recommended by Beer and Eshel (1985) assume that there is a sloping baseline in the absorbance spectrum as a result of particulate debris remaining within the crude extract. They also assume that the troughs (455, 592 and 645 nm) on either side of the PE and PC absorbance peaks (565 and 618 nm) are on that baseline (Beer and Eshel 1985; Figure 1). PE and PC contents were determined by using the absorbance values from the troughs to calculate the slope of the baseline and then subtracting the baseline value under the absorbance peak from the absorbance peak value. Therefore, if the slanted baseline resulted from the presence of particulate debris, one would expect that the slope of the baseline would decrease proportionally with greater centrifugation. Yet, when *Porphyra* extracts were centrifuged for increasing times/speeds, the absorbance values at 455 nm dropped faster than the 592 and 645 nm trough values and the distance between the “baseline” and the absorption peaks increased (Figure 2). Furthermore, no matter how long/fast the extracts were centrifuged, the trough absorption values (455, 592 and 645 nm) never reached zero (Figure 2). In fact, when *Porphyra* extracts were centrifuged until the spectrum remained unchanged (i.e., all of the particulate debris was removed) the “baseline” connecting the troughs (as per Beer and Eshel 1985) was not linear (Figure 2). The only wavelength region of the spectrum that reached the zero line was beyond 730 nm (Figure 2). Thus, the behavior of the particulate debris “absorbance” spectra was fairly linear and the slope decreased with centrifugation as predicted by Beer and Eshel (1985). However, these findings did not completely account for the behavior of the troughs in the crude extract (Figure 2).

Figure 2 Absorbance scan of *Porphyra purpurea* extract following increased centrifugation at 17,000 g. The dotted line represents the linear baseline described by Beer and Eshel (1985) following removal of particulate debris from the extract.

To further characterize the baseline, pelleted debris from crude extracts were resuspended following centrifugation treatments of 3, 5 and 30 min at 17,000 g and their spectral properties compared (Figure 3). It was apparent that the absorbance spectra of the pelleted debris remained fairly linear over the wavelengths used by Beer and Eshel (1985), yet the absorbance corresponding to the 455 nm trough dropped disproportionately faster than the 592 nm and 645 nm troughs (Figure 3). Additionally, as the samples were spun longer, the distance between the 592 nm trough and Beer and Eshel's (1985) "linear baseline" decreased. Thus, the middle trough was

Figure 3 Absorbance spectra of resuspended pellets of *P. purpurea* extracts following 3 min (solid line), 5 min (dashed line) and 30 min (dashed and dotted line) of centrifugation at 17,000 g. Diagonal lines represent the baseline behavior as described by Beer and Eshel (1985).

"pushed up" corresponding with the earlier observations of a pitched baseline (Figure 2).

Following these discoveries, it was evident that *Porphyra* extracts required sufficient centrifugation (to remove the suspended debris) prior to phycobilin quantification because the absorbance spectra changed disproportionately upon increased centrifugation (Figures 2 and 3). Furthermore, it became clear that Beer and Eshel's (1985) analyses were not adequate for this genus and a newly designed methodology for determining phycobilin content was needed that was specific for *Porphyra*.

Pigment composition of *Porphyra* extracts

Since the 455 nm and 592 nm troughs never reached zero or even the level of the 645 nm trough following centrifugation (Figure 2), the supernatant components were assumed to be void of particulate debris. That is, the remaining components of the supernatant should represent the sum of the individual spectra of each pigment in the solution. To characterize the absorbance spectrum of each dissolved pigment, supernatants reserved from thoroughly centrifuged extract samples were analyzed more carefully.

It was expected that the supernatant would contain R-PE and R-PC; however, it was not clear whether any additional pigments were contributing to the absorbance spectra of *Porphyra* extracts. Therefore, peaks of a *P. purpurea* absorbance spectrum were resolved using fourth derivative spectroscopy, which revealed seven distinct absorbance peaks (Figure 4). To identify what dissolved pigments corresponded to each peak, solutions of purified R-PE and R-PC were analyzed individually. Fourth derivative analysis of a pure R-PE absorbance spectrum (Figure 5a) resolved 2 peaks (Figure 5b) corresponding exactly with the third and fourth peaks from the *P. purpurea* extract (Figure 4). Similarly, fourth derivative analysis of a pure R-PC absorbance spectrum (Figure 5c) revealed a single peak (Figure 5d) that was consistent with the fifth peak of the *P. purpurea* extract (Figure 4).

The identification of only three of the seven fourth derivative absorption peaks necessitated further inquiry into the presence of extraneously dissolved pigments. Water soluble chlorophyll (Chl *d*; Chl *a* without a phytol tail) was initially considered because three of the seven peaks (1st, 2nd and 7th) corresponded with the known absorbance peaks of chlorophyll. When

Figure 4 Analysis of a *P. purpurea* crude aqueous extract following 30 min of centrifugation at 17,000 g. (a) Absorbance scan of the extract. (b) Fourth derivative analysis of the absorbance scan resolving seven distinct absorbance peaks (labeled 1–7).

the absorbance spectrum of chlorophyll extracted from *Ulva* sp. (Figure 5e) was analyzed by fourth derivative analysis (Figure 5f), peaks matching the first, second and seventh peaks of the *Porphyra* extract were resolved (Figure 4). The remaining sixth peak was determined to represent a pigment present in very small amounts (the height of the fourth derivative peak is inversely proportionate to the width of the absorbance peak, not its height) with a maximal absorbance of approximately 650 nm. Allophycocyanin (APC) fit these criteria and was later identified as the last peak of the *P. purpurea* absorbance scan. The identification of all peaks within *Porphyra* extracts elucidated the main components of *Porphyra* absorbance spectra: R-PE, R-PC and chlorophyll. However, whether or not the absorbance spectra of these pigments were overlapping had yet to be determined.

Developing new equations

Fourth derivative analyses of *Porphyra* aqueous extracts suggested that R-PE, R-PC and chlorophyll were the major components of the spectra. When a composite of these individual spectra was overlapped (Figure 6), it became evident why the 455 nm trough from *Porphyra* extracts never reached the zero “baseline” following centrifugation (Figure 3).

Figure 5 Absorbance and fourth derivative spectra of purified pigment solutions. (a) absorbance spectrum of R- phycoerythrin (R-PE). (b) 4th derivative analysis spectrum of R-PE. (c) Absorbance spectrum of R-phycocyanin (R-PC). (d) 4th derivative analysis spectrum of R-PC. (e) Absorbance spectrum of chlorophyll extracted from *Ulva* sp. (f) 4th derivative analysis spectrum of chlorophyll.

Figure 6 Absorbance spectra of a purified R-PE solution (solid line), a purified R-PC solution (dotted line) and a chlorophyll solution (dashed and dotted line) superimposed.

The absorbance spectrum of chlorophyll overlapped considerably with the absorbance spectrum of R-PE at 455 nm and was preventing that portion of the absorbance spectra from reaching zero (Figure 6). The presence of chlorophyll within the extract could have also accounted for the disproportional drop of the 455 nm trough (Figure 3). Namely, the rapid removal of non-water-soluble chlorophyll in the chloroplast debris during centrifugation would facilitate a faster decrease in 455 nm absorbance values compared to the 592 nm and 645 nm troughs. Furthermore, chlorophyll was found to affect the behavior of Beer and Eshel's (1985) "baseline" only because the absorbance of chlorophyll did not overlap with the absorption peaks of R-PE or R-PC (Figure 6). Similarly, because the absorption peak of R-PE did not overlap with the absorption peak of R-PC (Figure 7), the R-PC concentration could be calculated directly from the R-PC peak absorbance reading (618 nm).

Conversely, a portion of the purified R-PC solution absorbance was found to overlap with the absorption peak of purified R-PE (564 nm), causing the peak absorbance of R-PE within the R-PE + R-PC mixture to elevate (Figure 7). Thus, to compensate for the spectral interference of R-PC, the absorbance value at 564 nm needed to be corrected by an amount proportionate to the R-PC spectral overlap. It was determined that the R-PC absorbance at 564 nm was 45.83% of the peak R-PC absorbance (618 nm). Therefore, the true R-PE absorbance peak (564 nm) within

Figure 7 Individual absorption spectra of a purified R-PE solution (dashed line) and a purified R-PC solution (dotted line) superimposed with the absorption spectra of a solution mixture containing both purified R-PE and purified R-PC (solid line).

an extract containing both R-PE and R-PC could be determined by subtracting 45.83% of the R-PC absorbance value at 618 nm from the observed 564 nm absorbance value. Finally, because baseline values of zero were achieved only at wavelengths 730 nm and beyond, the absorbance values of each peak were determined by calculating the distance between the peak absorbance value and the absorbance value at 730 nm; the newly designated baseline.

The R-PC and R-PE contents (mg mL^{-1}) within aqueous extracts of *Porphyra* can be determined by multiplying the corrected absorbance values by the

Figure 8 Estimated R-PE and R-PC contents as calculated by the newly derived equations (solid line) and by Beer and Eshel's (1985) equation IV (dotted line) compared against the original *P. purpurea* absorbance scan (dashed line).

respective extinction coefficients (O'Carra 1965; Siegelman and Kycia 1978) in the following equations:

$$R - PC = 0.154(A_{618} - A_{730})$$

$$R - PE = 0.1247((A_{564} - A_{730}) - 0.4583(A_{618} - A_{730}))$$

Testing the new equations

To compare the effectiveness of our new equations against those recommended by Beer and Eshel (1985), extracts from *P. purpurea* were centrifuged until no observable changes in absorbance occurred. The R-PE and R-PC contents of the sample were determined via the newly derived equations and by the recommended equations of Beer and Eshel (1985). The estimated R-PE and R-PC contents were higher using the new equations (0.091 and 0.098 mg mL⁻¹, respectively) compared to the contents estimated via Beer and Eshel's recommended equations (0.072 and 0.044 mg mL⁻¹, respectively). Mixtures of pure R-PE and pure R-PC were constructed (in concentrations corresponding to the estimates calculated by each set of equations) and the absorbance spectrum of each was recorded. A comparison of phycobilin absorption spectra as calculated by the newly derived equations and those of Beer and Eshel's (1985) clearly indicated that the latter equations underestimate the phycobilin contents of *Porphyra* samples (Figure 8).

Acknowledgements This work was funded in part by the USDA New Hampshire Agricultural Experiment Station (NHAES) as Hatch grants #NH00404 and NH00439 and is scientific contribution number 2303 from the NHAES. We would like thank Dr. Leland S. Jahnke, Dr. Arthur C. Mathieson and especially reviewer SB for helpful comments on the manuscript.

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