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Isolation and characterization of R-phycoerythrin subunits and enzymatic digests

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Abstract

Subunits and enzymatic digests of the highly fluorescent phycobiliprotein R-phycoerythrin (R-PE) were analyzed by several separation and detection techniques including HPLC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), CE, and HPLC–electrospray ionization (ESI) MS. R-PE subunits were isolated by HPLC and detected as single molecules by total internal reflection fluorescence microscopy. The results show efficient absorption and fluorescence of the R-PE subunits and digest peptides, originating from the incorporation of phycoerythrobilin and phycourobilin chromophores in them. In addition, HPLC–ESI-MS and SDS–PAGE were optimized to determine the molecular masses of phycobiliprotein subunits and the chromophore-containing peptides, as well as the amino acid sequences of the latter. Favorable spectroscopic and structural properties of R-PE subunits and enzymatic digests, even under denaturing conditions, make these molecules suitable for use as fluorescence labels for biomolecules.

Keywords: Total internal reflection fluorescence microscopy; Detection, LC; Phycoerythrin; Phycobiliproteins; Proteins

1. Introduction

Research on fluorescent proteins has been intensive, especially after the cloning of green fluorescent protein (GFP) from jellyfish *A. victoria* in different types of cells [1]. Recently, new fluorescent proteins from the GFP family were discovered in corals [2]. Phycobiliproteins are found in cyanobacteria and several groups of eucaryotic algae (red algae, criptomonads and glaucophytes) [3]. These organisms contain phycobilisomes, phycobiliprotein complexes that have an important role in the photosynthesis. Although they cannot be cloned due to complexity of their structures, phycobiliproteins remain as very useful fluorescent probes due to their excellent spectroscopic properties [4,5].

Phycobiliproteins consist of three groups: allophycocyanins, phycocyanins and phycoerythrins [3]. They are all composed of two subunits (α and β), while the third subunit (γ), a linker peptide, is found in phycoerythrins. The structure of R-phycoerythrin (R-PE) and B-phycoerythrin (B-PE) can be described as $(\alpha\beta)_6\gamma$, while phycocyanin (PC) and allophycocyanin (APC) have the structure $(\alpha\beta)_3$ [4]. Each subunit contains one or more phycobilin chromophores (phycobilins) bound to specific cysteines in the polypeptide chains by thioether bonds. The outstanding absorption and fluorescence properties of phycobiliproteins in the visible region originate from phycobilins and their interactions within polypeptide chains [4,5]. Eight different phycobilins were found in phycobiliprotiens [6]. The most representative phycobilins are phycoerythrobilin (PEB), phycourobilin (PUB), phycocyanobilin (PCB) and phycobiliviloin (PXB) (Fig. 1). PEB is found in C-phycoerythrin (C-PE), R-PE, B-PE and PC, PUB is found in R-PE and B-PE, PCB is found in PC, APC, phycoerythrocyanin (PEC) and phycoerythrins, while PXB is found in PEC [3,7]. The number of phycobilins and the phycobiliprotein structure depend on the species of origin, but some phycobilin-binding sites were conserved during phycobiliprotein evolution [3,7].

R-PE from red algae *G. coulteri* has the structure $(\alpha\beta)_6\gamma$ and molecular mass (M_r) of ∼240,000 [8]. Two γ subunits

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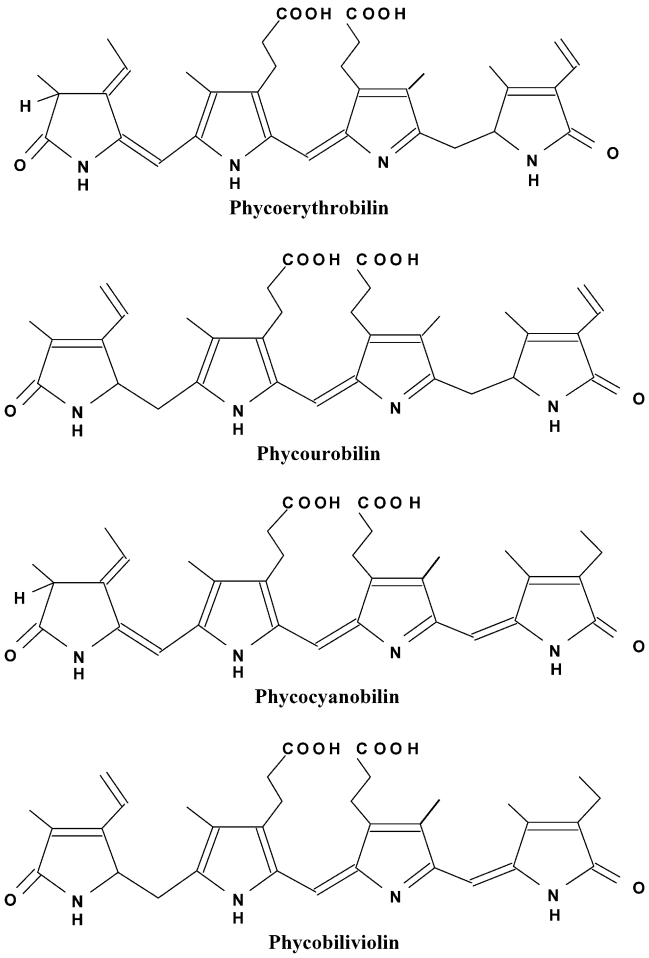

Fig. 1. Structures of four representative phycobilins: phycoerythrobilin (PEB), phycourobilin (PUB), phycocyanobilin (PCB) and phycobiliviolin (PXB).

differing in amino-acid sequences were found in this protein. The α subunit ($M_r \approx 17{,}000$) contains two PEB chromophores; the β subunit ($M_r \approx 18{,}000$) contains two PEB and one PUB chromophore, while the γ subunits (M_r values $\approx 30{,}000$) contain three PUBs and one PEB chromophore [8]. Two or three γ subunits and different chromophore contents were found in R-PE from other red algal species [9,10]. The absorption spectrum of R-PE shows maxima at 496 and 565 nm due to the presence of PUB and PEB chromophores, respectively. This was confirmed by spectral characterization of R-PE chromophores and peptide digests [8,11,12]. The single fluorescence maximum of R-PE at 580 nm is a consequence of fluorescence resonance energy transfer (FRET) from PUB to PEB chromophore [13].

Phycobiliproteins conjugated to antibodies are used in numerous fluorescence assays [4,5]. Due to their stability, high absorption coefficients and high quantum yields, B-PE and R-PE have been detected as single-molecules [14–16] and imaged by total internal reflection fluorescence microscopy (TIRFM) on a fused-silica prism [16]. Also, R-PE has been tracked by fluorescence video microscopy in the cytoplasm and nucleoplasm of a single mammalian cell [17].

Sequences of many phycobiliprotein apo-subunits have been obtained by sequencing cyanobacterial and algal genomes [7]. However, characterization of phycobiliprotein subunits and their enzymatic digests is necessary to elucidate phycobiliprotein composition and chromophore content. HPLC, gel filtration, ion-exchange chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) have been used for the analysis of phycobiliprotein subunits and enzymatic digests. A universal-reversed-phase (RP)-HPLC gradient consisting of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 2:1 (v/v) acetonitrile:isopropanol on a C_4 column was used to separate and characterize subunits of diverse phycobiliproteins [18]. Fast performance LC (FPLC) and SDS–PAGE were used to separate γ subunits of R-PE from red algae *C. corymbosum* and *A. sparsum* [9]. α and β subunits of C-PE from cyanobacteria *Pseudanabaena* W 1173 were separated by gel filtration and ultracentrifugation, and their absorption coefficients and quantum yields were determined [19]. SDS–PAGE followed by Coomassie staining and HPLC was used for the separation of C-PE and APC subunits isolated from cyanobacteria *S. platensis* [20]. B-PE isolated from the unicellular red algae *P. cruentum* was characterized by SDS–PAGE and by RP-HPLC, using 0.1% TFA in water and 0.1% TFA in acetonitrile on a C_4 column [21]. Recently, RP-HPLC–electrospray ionization (ESI) MS, using also 0.1% TFA in water and 0.1% TFA in acetonitrile on C_4 column, was used to analyze C-PC and APC subunits from phycobilisomes of cyanobacteria *Synechocystis* 6803 [22,23].

Separation and purification of phycobiliprotein enzymatic digest peptides were performed by gel filtration, ion-exchange chromatography and HPLC, to sequence them and to find the phycobilin-binding sites in B-PE and R-PE [8,24,25]. Secondary-ion mass spectrometry was used to determine molecular masses and to confirm the sequences of chromophore-containing peptides [26,27].

Although CE with laser-induced fluorescence (LIF) was used to study phycobiliproteins [28], there have been no reports on using CE for the separation of R-PE subunits and enzymatic digests. One specific way to detect phycobilin-bound proteins and peptides in polyacrilamide gels is SDS–PAGE in the presence of zinc acetate [29]. After excitation by UV light, orange fluorescence of phycobiliprotein-zinc complexes in gels or membranes is detected. This method is comparable in sensitivity to commonly used Coomassie gel staining.

In this research, HPLC, SDS–PAGE and CZE with absorption and fluorescence detections were used to analyze R-PE subunits and enzymatic digests. The goal is to isolate these polypeptides, determine their spectroscopic properties and evaluate their suitability for single-molecule detection. In addition, we develop HPLC–ESI-MS and SDS–PAGE methods to determine the molecular weights of phycobiliprotein subunits and peptide digests, as well as the amino acid sequences of the chromophore-containing peptides.

2. Experimental

2.1. Chemicals

Phycobiliproteins (R-PE, B-PE and APC) were purchased as solutions in phosphate buffer (C = 4 mg/ml, pH = 7.4) from Molecular Probes (Eugene, OR, USA). R-PE was from red algae *P. tenera*, B-PE was from red algae *P. cruentum*, and APC was from cyanobacteria *A. variabilis*. HPLC-grade acetonitrile, dimethyl sulfoxide (DMSO), 88% formic acid, methanol, mercury(II) chloride and urea were from Fisher Scientific (Pittsburgh, PA, USA). TFA, Tris base, sodium phosphate, ammonium hydrogen carbonate, pepsin and trypsine were from Sigma (St. Louis, MO, USA). Milli-Q nano-pure water and Sep-Pak cartridges were from Waters–Millipore (Milford, Boston, MA, USA). SDS–PAGE chemicals were from Bio-Rad (Richmond, CA, USA).

2.2. Sample preparation

Protein solutions were centrifuged in the microcentrifuge at 5000 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in water just before the experiments. For pepsin digestion of R-PE, 30 μl of R-PE (C = 1 mg/ml) was centrifuged as above. Then 9 μl of pepsin solution (C = 2 mg/ml) and 90 μl of 0.025 M HCl was added. The mixture was heated at 37 °C for 4 h. The procedure for trypsin digestion was adopted from reference [8]. Twenty-five μl of R-PE (C = 1 mg/ml) was centrifuged as above. Seventy-five μl of 0.025 M HCl was added. One μl of trypsin (C = 1 mg/ml) and 1 mg of NH_4HCO3 were added and the mixture was heated at 37 °C for 2 h. Then, a new 1 μl aliquot of trypsin solution was added and incubation continued at 37 °C for two more hours.

PEB was a generous gift of Max Storf (University of Munich, Germany). An urobilin product, presumably PUB, was isolated from B-PE using a modified procedure for PEB isolation [30]. 2 mg of B-PE was refluxed in 100 ml of $HgCl_2$ solution in methanol (C = 2 mg/ml) at 45 °C for 16 h. After centrifugation at 17,000 × g for 20 min, 200 μl of 2-mercaptoethanol was added, and the solution was centrifuged again as above. The methanol solution was evaporated under vacuum and the remaining supernatant was cleaned by solid-phase extraction through a C_{18} Sep-Pak cartridge.

2.3. HPLC separations with absorption and fluorescence detection

A Shimadzu Class VP HPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) was used for HPLC separations on an analytical C_4 column (250 mm × 4.6 mm, Vydac, Hesperia, CA, USA). The instrument was equipped with both photodiode array (PDA) UV–vis and spectrofluorimetric detectors. For subunit separations, 10 μl of R-PE solution (C = 4 mg/ml) was injected on the column previously equilibrated with 75% of phase A (0.1% TFA in water) and 25% of phase B (0.1% TFA in acetonitrile). The flow rate was 0.8 ml/min. A gradient from reference [21] was used. Later it was found that following gradient gives baseline separation as well: 0–40 min 75% of B, 40–45 min 95% of B, and 45–50 min 25% of B. The PDA detector was set with both deuterium and tungsten lamps on to monitor the absorbance of the eluent from 190 to 800 nm. For subunit absorption coefficient determination, four R-PE solutions with concentration of 1, 2, 3 and 4 mg/ml were prepared and run in a sequence using the above gradient.

For fluorescence detection, the excitation wavelength was set at 496 nm and the emission wavelength was set at 580 nm. To measure excitation and emission spectra of the subunits on-line, pumps were stopped during the rising edge of the absorbance and fluorescence peaks, and a wavelength scan was acquired. Subunits were collected manually as they elute out of the column for subsequent fluorescence measurements.

For separations of pepsin and trypsine digests, 20 μl of digests were injected on the C_4 column equilibrated with 95% of phase A and 5% of phase B. Separations were done according to the following gradient: 0–15 min 20% B, 15–45 min 35% B, 45–55 min 45% B and 55–65 min 95% B. The eluent was monitored as in the case of the subunits.

2.4. Fluorescence and absorption spectrometry

A luminescence spectrometer LS50B (Perkin-Elmer, Beakonsfield, UK) was used to measure fluorescence spectra of the collected subunits and chromophores in increments of 0.5 nm. Widths of the excitation and emission slits were set at 10 nm. Absorbance spectra of chromophores were measured by a 8452A diode-array spectrophotometer (Hewlett Packard, Palo Alto, CA, USA) in increments of 2 nm.

2.5. TIRFM

Subunits, collected after HPLC separation, were diluted one thousand times by phosphate buffer (pH = 9). Five μl of a subunit solution was set on a 22 mm square cover slip (Corning, New York, NY, USA) and put on a fused-silica prism (Melles Griot, Irvine, CA, USA). A Coherent, Innova 90 (Santa Clara, CA, USA) 488-nm argon-ion laser was used to excite R-PE subunits. Fluorescence was collected by a 40× immersion-oil Plan-Neofluor objective, NA = 1.3 (Carl Zeiss, Thornwood, NY, USA) through type FF immersion oil (Cargille, Cedar Grove, NJ, USA) with a refractive index of n = 1.48. Fluorescence images were taken by a cascade intensified charge-coupled device (ICCD, Roper Scientific, Trenton, NJ, USA) with a pixel size of 7.5 μm × 7.5 μm. The camera chip was kept at –35 °C by thermoelectric cooling. A 488-nm holographic notch filter (Kaiser Optical System, Ann Arbor, MI, USA) with an optical density of >6 was placed between the objective and ICCD camera to prevent stray light from reaching the ICCD. The exposure times for the ICCD camera and the laser shutter were synchronized by a shutter driver/timer, Uniblitz ST132 (Vincent Associates, Rochester,

NY, USA). The digitization resolution of the camera was 16 bit. The digital-analog converter (DAC) setting was 3689. The data rate was 2 Hz (0.5 s/frame). The exposure time for each frame was 20 ms. The frame transfer of the ICCD camera was operated in the external synchronization mode. A sequence of frames were acquired for each sample via V++ software (Roper Scientific). All frames were analyzed off-line.

2.6. CE separations with absorption and fluorescence detection

A Beckman PACE/MDQ CE instrument (Beckman Coulter, Carlsbad, CA, USA) equipped with a UV–vis PDA detector and a 488-nm argon-ion laser-induced fluorescence (LIF) detectors was used for separation and detection on 75 μm i.d. fused-silica capillaries (Polymicro, Phoenix, AZ, USA). Samples were injected hydrodynamically using pressure of 3447 Pa for 5 s. A voltage of 25 kV was applied on the 60 cm long capillary. For absorbance measurements, the PDA detected signal from 190 to 600 nm. For LIF detection, a 488-nm notch filter and a 580-nm band-pass emission filter were used. Separation of the subunits was accomplished in 50 mM phosphate buffer containing 4.5 M urea (pH = 2) within 90 min. Trypsin digest was separated in 0.1 M phosphate buffer (pH = 2.5) in 60 min. After pretreatment of the capillary with 0.1 M NaOH for 5 min, pepsin digest was separated in 0.1 M Tris buffer (pH = 7.6) for 15 min.

2.7. SDS–PAGE analysis

SDS–PAGE equipment was from Bio-Rad. Ten μl of phycobiliproteins (C = 4 mg/ml) was centrifuged at 5000 rpm for 10 min. Forty μl of water, 47. 5 μl of Laemli sample buffer and 2.5 μl of 2-mercaptoethanol were added and the mixture was heated for 10 min at 95 °C. Thirty μl of the mixture and 5 μl of molecular mass standards were loaded on the 12% polyacrylamide (PA) Tris–HCl gel and were separated using a current of 30 mA. After separation, the gel was washed three times for 5 min each in water, and scanned by a fluorescence imager Typhoon 8600 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a 532-nm laser for excitation, 580BP30 filter for emission, and a detector voltage of 600 V. After fluorescence detection, the gel was stained with Bio-safe Coomassie blue for 1 h and washed in water for 30 min.

2.8. HPLC/ESI-MS experiments

A Shimadzu LCMS-2010 instrument equipped with a dual-channel UV–vis detector was used for the HPLC–ESI-MS experiments. 3.7% (v/v) formic acid in water (A) and 3.7% (v/v) formic acid in acetonitrile (B) were used as solvents. Separations were performed on a Vydac C_4 HPLC column (250 mm × 2.1 mm) using a flow rate of 0.1 ml/min. Mass spectra were collected in the positive-ion mode using the LCMS Solution Main Program. The following values were set in the tuning file: CDL temperature 250 °C, nitrogen gas flow 4.5 l/min, block temperature 200 °C, probe voltage 4.5 kV, CDL voltage 25 V, Q array voltage 30.0 V/25.0 V/55.0 V, and Q array RF 150.00. The dual UV–vis detector was set to monitor absorption of subunits and chromophore-containing peptides at their respective absorption maxima (496 and 555 nm in the case of R-PE and B-PE subunits, 650 nm for APC subunits, and 496 and 550 nm for R-PE digest peptides). Molecular mass of subunits and chromophore-containing peptides were determined using LCMS Profile Post Run program.

In the case of subunits separation, 15 μl of phycobiliproteins (C = 4 mg/ml) was loaded on the column after equilibrating with 75% of phase A and 25% of phase B. The gradient used for the separation of R-PE and B-PE subunits and their mixture was: 0–40 min 75% B, 40–45 min 95% B and 45–50 min 25% B. For separation of APC subunits and their mixture with R-PE and B-PE subunits the following gradient was used: 0–60 min 75% B, 60–70 min 95% B, and 70–80 min 25% B. The scan range of the MS detector was 850–1650 m/z with a scan interval of 5 s, scan speed of 500 and detector gain of 1.5 kV.

For separations of R-PE digests, 20 μl of pepsin or trypsin R-PE digest was injected on the column after equilibrating with 5% B. The scan range of MS detector was 300–1800 m/z with a scan interval of 5 s, scan speed of 500 and detector gain of 1.5 kV. For pepsin digest, the gradient used was 0–15 min 20% B 15–75 min 30% B 75–90 min 95% B, and 90–100 min 5% B. For trypsin digest separation the following gradient was used: 0–15 min 20% B, 15–40 min 30% B, 40–55 min 60% B, 55–80 min 95% B, and 80–90 min 5% B. The measured M_r values of subunits and peptides were compared with their respective MWs found in the Swiss-Prot and TrEMBL protein database [7] and calculated by the program PAWS (ProteoMetrics, New York, NY, USA).

3. Results and discussion

3.1. Isolation and spectroscopic characterization of R-PE subunits

R-PE subunits were baseline separated by HPLC (Fig. 2). The elution profile looks similar as in earlier HPLC separations of R-PE and B-PE subunits [18,20]. Assignment of subunits was achieved from their absorption spectra (inset of Fig. 2 and Table 1). The α subunit (t_R = 24.8 min) shows an absorption maximum at 555 nm due to the presence of PEB chromophore, while the β subunit (t_R = 30.4 min) and γ subunits (t_R =17.0 min and t_R = 18.2 min) show maxima at both 496 and 555 nm due to the presence of both PUB and PEB chromophores [4,8,18]. Chromophore content of R-PE subunits is characteristic for R-PE from *P. tenera* and several other species [7]. As in the work of Bermejo et al on HPLC separation of B-PE subunits [21], HPLC separation of R-PE subunits was semi-preparative. Subunits were manu-

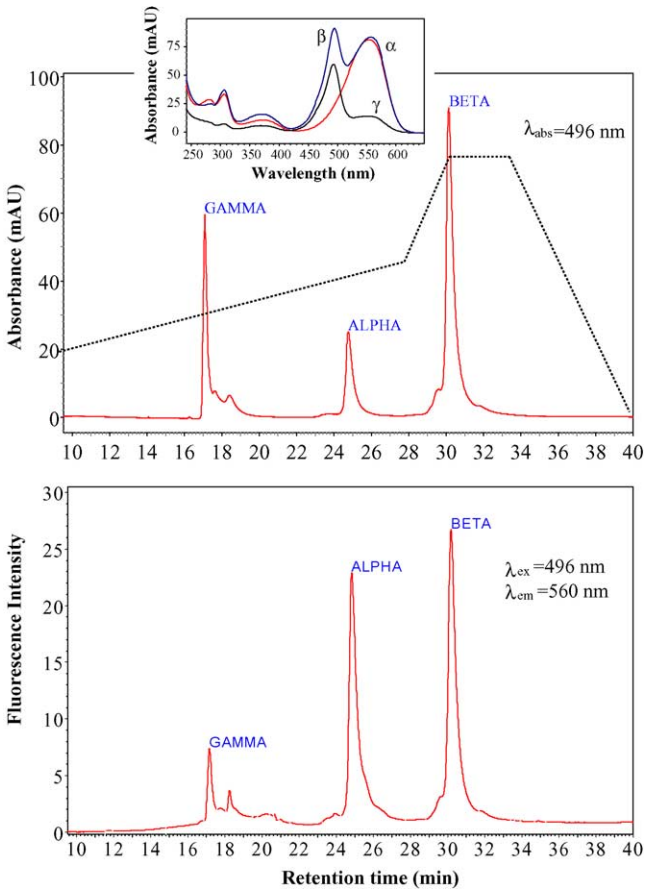

Fig. 2. HPLC separation of R-PE subunits. Chromatograms were simultaneously recorded by a UV–vis PDA detector (top) and a spectrofluorometric detector (bottom). Peaks were assigned according to the subunit absorbance spectra (inset). Dotted line represents the HPLC gradient in percentage of acetonitrile with 0.1% TFA [21].

ally collected for further analysis (fluorescence spectroscopy and fluorescence microscopy).

Subunit absorption coefficients were determined from the calibration curve of on-line subunit absorbance versus subunit concentration. Slight adsorption of the subunits on the column stationary phase occurred (approximately 5% of the amount injected on the column). Because of that, a correction was introduced for subunit absorbance. Values of ∼10,000 cm^{-1} M^{-1} were found for absorption coefficients of α subunit (at 555 nm) and β subunit (both at 555 and 496 nm). R-PE subunits were diluted in the detection cell of the HPLC UV–vis PDA detector, so that exact values of absorption coefficients at pH of 2 should be higher than determined values. Because the subunits were denatured in the mobile phase, absorption coefficients of "native" R-PE subunits should be much larger than 10,000 cm^{-1} M^{-1}. Absorption coefficients of C-PE α and β subunits were determined to be 151,300 and 266,200 cm^{-1} M^{-1} at their respective absorption maxima (566 and 557 nm) in phosphate buffer at pH = 7 [19]. Since the structure and chromophore content of C-PE [31] is similar as structure of R-PE it is reasonable to expect approximately the same absorption coefficients of R-PE subunits.

Table 1
Spectroscopic characteristics and molecular weights of R-PE subunits compared with R-PE and its chromophores

	λ_{abs}^{max} (nm)	λ_{ex}^{max} (nm)	λ_{em}^{max} (nm)	M_γ
R-PE	496, 565	496, 565	575	240000
PUB	494	494	505	590.72
PEB	592	592	626	586.69
α subunit	555	544	564	18888
β subunit	496, 555	496, 557	504, 565	20304
γ subunits	496, 555	496, 557	504, 565	30168

Molecular mass of R-PE was taken from the literature [5]. Molecular masses of phycobilins were calculated from their molecular formulas. Molecular mass of one of the γ subunits was determined.

Despite the low pH used for separation, all subunits show high fluorescence (Fig. 2, lower chromatogram). Excitation and emission spectra of R-PE subunits were recorded by spectrofluorometric HPLC detector and fluorescence spectrometer (Fig. 3), and the fluorescence maxima are listed in Table 1. Excitation spectra of subunits show maxima at approximately the same wavelengths as their respective absorbance spectra. The α subunit emission spectrum has a single emission maximum at 564 nm, while the β and γ subunits show emission maxima both at 504 and ∼565 nm. Peaks at 505 and 565 nm are due to fluorescence of covalently bound PUB and PEB chromophores respectively, as confirmed by the absorption and fluorescence spectra of free chromophores (Table 1). The absorption and fluorescence maxima of PEB chromophore show hypsochromic effect when bound to the polypeptide chain due to decreased conjugation of the double bonds.

The quantum yields of subunits could be determined by the fluorescence spectrometer using the method of Parker and Rees [32]. If the fluorescence spectra of two compounds are measured with the same instrument at the same excitation wavelength, the ratio of fluorescence intensities is given by the ratio of the spectral areas. For this measurement to be used with an HPLC detector it is necessary to have a standard compound with known quantum yield at the pH value used for separation. Unfortunately, there were no such standards. Quantum yields of 0.51 and 0.56 were found for C-PE α and β subunits at pH = 7 based on measurements in the fluorescence spectrometer [19].

It is interesting to try to separate R-PE subunits by capillary zone electrophoresis (CZE). CZE separations followed by LIF detection showed several fluorescent peaks (Fig. 4). Fluorescent subunits show also absorbances at 496 and 555 nm (data not shown), but the CE PDA detector could not reconstruct the absorption spectra of the subunits. So, the assignment of these peaks to specific subunits was not possible. Considering the amount of sample injected, the high fluorescence of R-PE subunits is obvious. CE subunit separation times were relatively long and the reproducibility was inferior. From the amino acid sequences of R-PE subunits from *P. tenera* [7], we calculated the p*I* values to be 5.40 and 6.23 for α and β subunits, respectively. So, subunits are positively charged at the pH used for CE separation and adsorption on the negatively charged capillary wall is

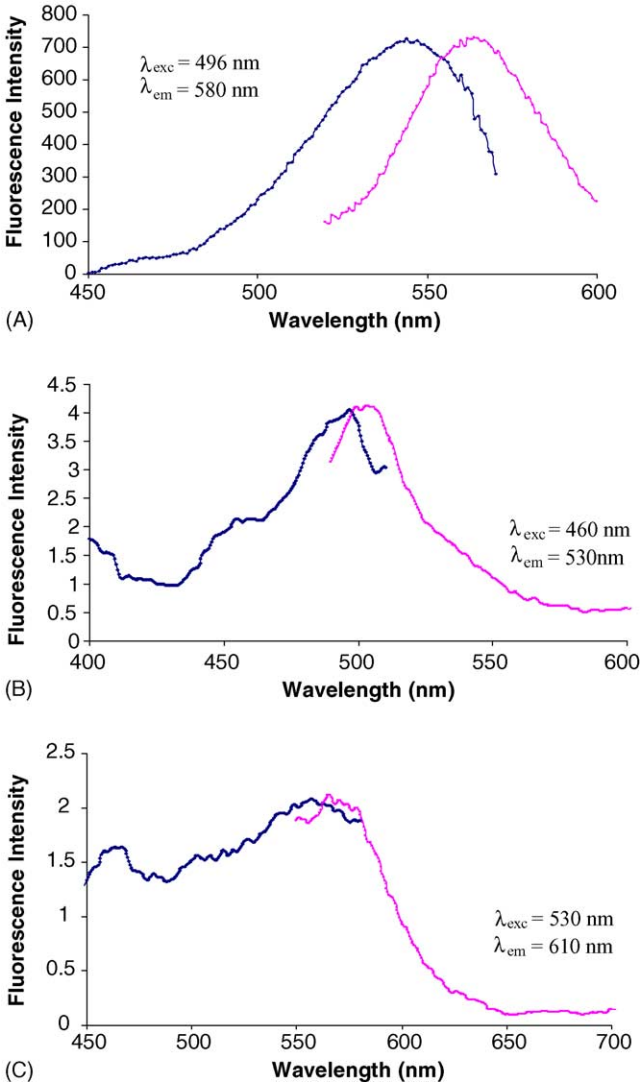

Fig. 3. (A) Normalized excitation and emission fluorescence spectra of R-PE α subunit recorded by the HPLC spectrofluorometric detector. (B,C) Normalized excitation and emission fluorescence spectra of R-PE β subunit recorded in a fluorescence spectrometer. The γ subunit fluorescence spectra are same as the β subunit spectra.

significant. Adsorption could be prevented and separation improved if coated capillaries were used.

3.2. Separation and spectroscopic characterization of R-PE enzymatic digests

RP-HPLC separation of trypsin and pepsin digests (Fig. 5) was achieved on a C_4 column. Separations on the C_4 column were more efficient than separations on C_8 and C_{18} columns due to the hydrophobicity of phycobilin-containing peptides. Peptides having retention times from 20 to 25 min show absorption maxima at 496 nm (left inset of Fig. 5), and correspond to PUB-containing peptides. Peptides eluting from 25 to 34 min correspond to PEB-containing peptides and have absorption maxima at 550 nm (right inset of Fig. 5). Both

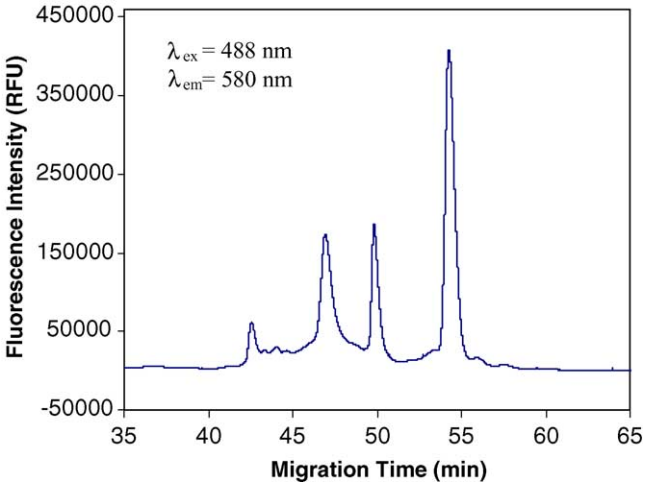

Fig. 4. CZE separation of R-PE subunits.

PEB peptides (Fig. 5, lower) and PUB containing peptides are fluorescent because of the chromophore. R-PE pepsin and trypsin digests were also separated by CZE. Fig. 6 shows the CZE separation of R-PE pepsin digest followed by LIF detection. Fluorescence of PEB-containing peptides is evident and the separation reproducibility was very good.

3.3. Single-molecule detection of R-PE subunits by TIRFM

Single-molecule experiments confirm that R-PE subunits are highly efficient in absorption and in fluorescence. After isolation by HPLC, R-PE subunits were seen as individual molecules in the phosphate buffer (pH = 9) by TIRFM (Fig. 7). From the amount of R-PE on the column, it was calculated that the concentrations of imaged subunits were around 1 nM. If the thickness of the excitation zone in TIRFM is 200 nm and the size of the imaging window (200 pixels × 200 pixels) is 2.25 mm^2, the number of molecules present in the volume of 4.5×10^{-4} mm^3 after magnification with 40× objective should be around 200. We observed 198, 498 and 297 molecules for the α, β and γ subunits, respectively. The β and γ subunits are brighter than the α subunit (Table 2) due to more efficient excitation of β and γ subunits at 488 nm. This wavelength is also close to the excitation maximum of the PUB chromophore. If R-PE subunits are imaged in the HPLC mobile phase (pH ≈ 2.0), they aggregated and became permanently adsorbed on the glass surface (shown for the α

Table 2
Signal-to-background ratios in single-molecule experiments

Subunit	Signal (s)	Background (bg)	Background deviation (d)	(s-bg)/d
R-PE α	15000	8600	1307	4.90
R-PE β	30000	9900	1498	13.42
R-PE γ	20000	10770	1622	5.69

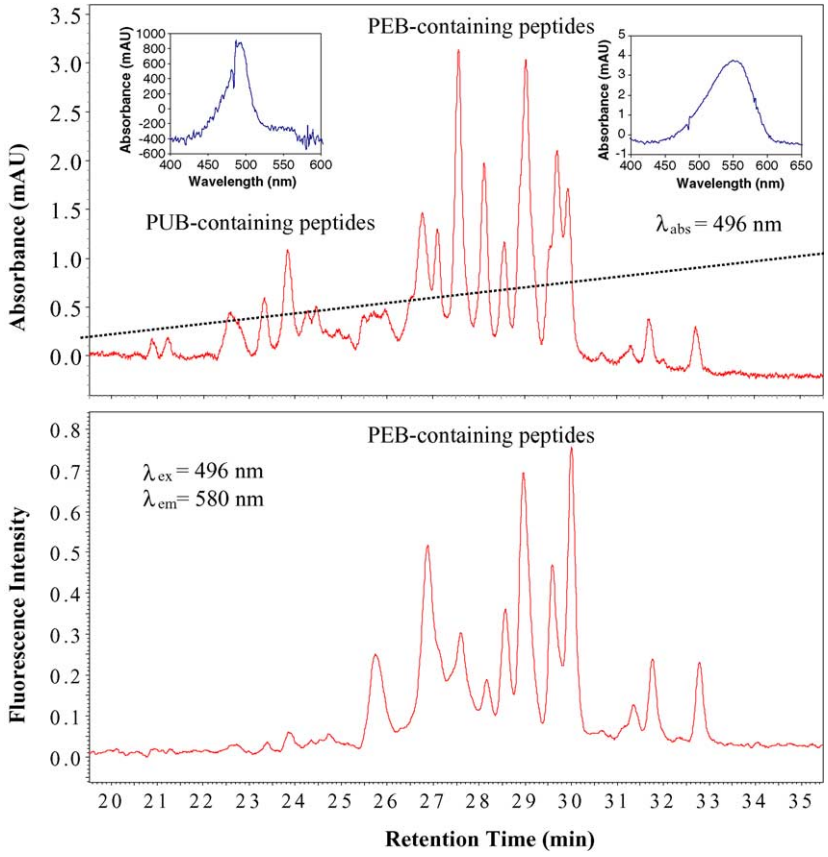

Fig. 5. HPLC separation of chromophore-containing peptides. Chromatograms were simultaneously recorded by a UV–vis PDA detector (top) and a spectrofluorometric detector (bottom). Dotted line represents the HPLC gradient in percentage of acetonitrile with 0.1% TFA (for gradient details see Section 2.3).

subunit in Fig. 7). Subunits are positively charged at low pH and are attracted to the negatively charged silica prism, confirming the adsorption effect seen during CZE separation. Same type of correlations between adsorption of proteins in TIRFM and during CZE was noticed for R-PE molecules [16].

3.4. SDS–PAGE analysis of phycobiliprotein subunits

To gain further insights into the properties and structures of R-PE subunits and enzymatic digests, SDS–PAGE and HPLC–ESI-MS were employed. Coomassie-stained gel after SDS–PAGE analysis (Fig. 8, left) shows one band corresponding to overlapped α and β subunits ($M_r \approx 20{,}000$) and two low-intensity bands corresponding to two different gamma subunits ($M_r \approx 30{,}000$). SDS–PAGE analysis followed by fluorescence detection also showed two other bands with M_r values between 40,000 and 50,000 (Fig. 8, right). These bands could correspond to either non-denatured subunit aggregates or the structurally different gamma subunits. SDS–PAGE was used for separation of B-PE and APC as well. For separation of B-PE subunits, the bands look similar to those for the R-PE subunits due to structural similarities of B-PE and R-PE. Improved resolution of the subunits from these proteins could probably be achieved if higher percentage PA gels are used. α and β subunits of B-PE from *P. cruentum* were separated by SDS–PAGE on 16.5% polyacrylamide gel [21]. In the case of APC two bands with respective M_r

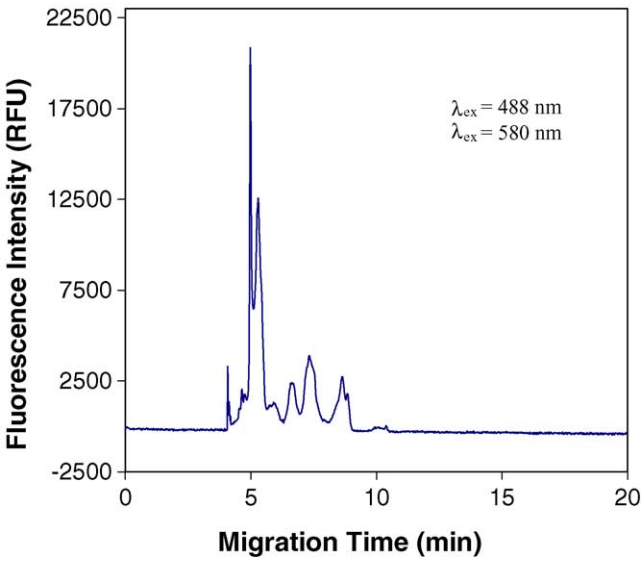

Fig. 6. CZE separation of R-PE pepsin digest.

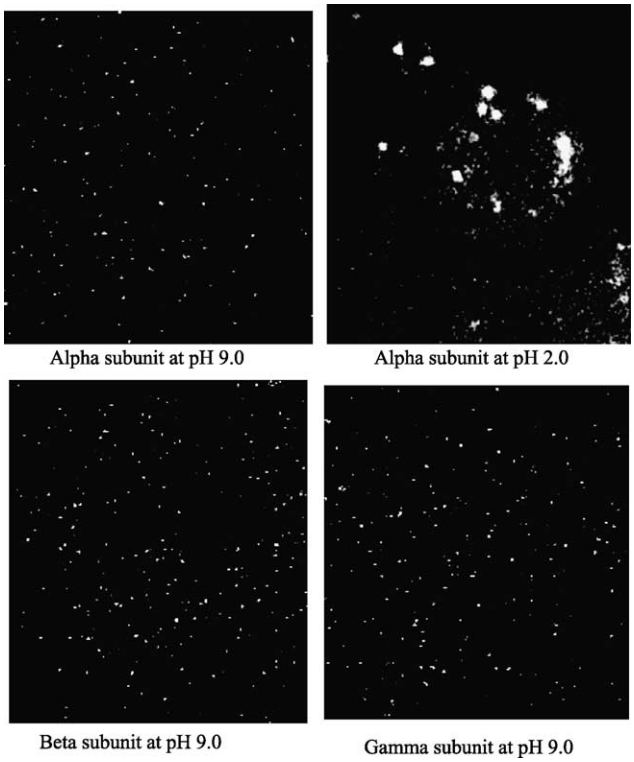

Fig. 7. Images of R-PE subunits taken by TIRFM. Each spot represents a single molecule.

values of approximately 16,000 and 18,000 (Fig. 8) are seen in both Coomassie and fluorescence detection. These bands correspond to APC α and β subunits respectively. The fluorescence gel imager has been successfully used for imaging phycobiliproteins after native PAGE [33]. Here we show that this instrument can also be used for the detection of phycobiliprotein subunits on the denaturing gel. This method is more sensitive but is less selective for the detection of bilin-bound proteins than UV-excited fluorescence of zinc-phycobiliprotein complexes [29]. The method could also be used to detect phycobilin-bound peptides in gels or on membranes.

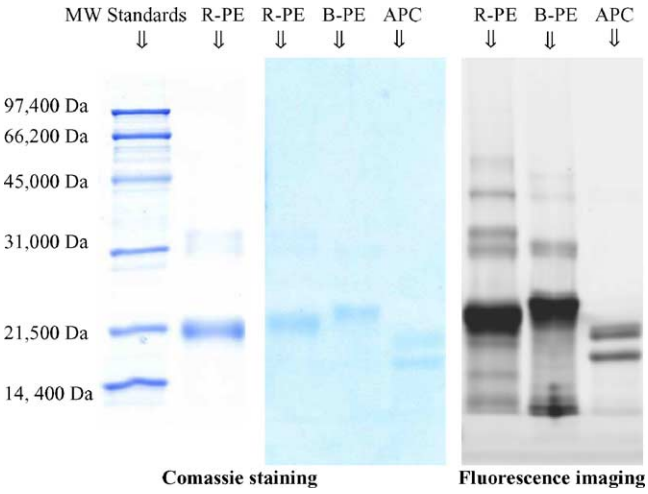

Fig. 8. SDS–PAGE separation of R-PE, B-PE and APC subunits followed by Coomassie staining (left) and fluorescence detection (right). Da = dalton.

3.5. HPLC–ESI-MS analysis of phycobiliprotein subunits

HPLC–ESI-MS separation of R-PE subunits was achieved (Fig. 9). An unusually high content of formic acid was used during the separation to match the pH of the mobile phase when 0.1% of TFA was used. Formic acid (HCOOH) is used in LC–ESI-MS to replace TFA because it makes weaker complexes with the ions of interest than TFA and improves the ion signal [34]. While usually up to 1% (v/v) of formic acid was used for HPLC–ESI-MS separations [34,35], we used 3.7% (v/v) formic acid in both water and acetonitrile. HPLC separation of α and β subunits is achieved efficiently, while separation of γ subunits is even better than in the case when 0.1% TFA was used in the mobile phase. Up to four peaks corresponding to R-PE gamma subunits could be seen (Fig. 9). Mass spectra of α, β and one of γ subunits (t_R =17.5 min) show multiply charged ions (insets in Fig. 9). Subunits' M_r values were calculated from these mass spectra (Table 1).

To check if HPLC–ESI-MS could be used for the analysis of other phycobiliproteins, B-PE and APC subunits were separated (Fig. 10). The measured M_r values of B-PE α and β subunits are 18,977 and 20,327, respectively (Fig. 11). Three values (17,928, 17,824 and 16,763) were obtained from ESI mass spectrum of the APC α subunit (Fig. 11), probably due to cluster formation with formic acid molecules or slight changes in the structure of this subunit at low pH. The nominal M_r of the APC β subunit is 17,846. R-PE, B-PE and APC subunits were separated from their respective mixtures, what could make this HPLC–ESI-MS method useful for analysis of phycobilisomes of algae or cyanobacteria [22,23]. Also, M_r values of the subunits provide the possibility to determine the number of the chromophores on a subunit if the sequence of an apo-subunit is known. The measured M_r values are in excellent agreement with M_r values of the phycobiliprotein subunits from *P. tenera*, *P. cruentum* and *A. variabilis* found in Swiss-Prot and TrEMBL protein database (Table 3 and [7]). These values are more accurate than M_r values of R-PE subunit found by SDS–PAGE (Section 3.4).

Table 3
Comparison of measured (by HPLC–ESI-MS) and calculated M_r values of phycobiliprotein subunits

Subunit	*P. tenera*		*P. cruentum*		*A. variabilis*
	R-PE α	R-PE β	B-PE α	B-PE β	APC β
Measured M_r	18888	20304	18977	20327	17846
Calculated M_r	18839	20201	18990	20332	17779

M_r values were calculated from amino acid sequences, phycobilin content, and post-translational modifications of respective phycobiliprotein subunits found in Swiss-Prot and TrEMBL protein database [7].

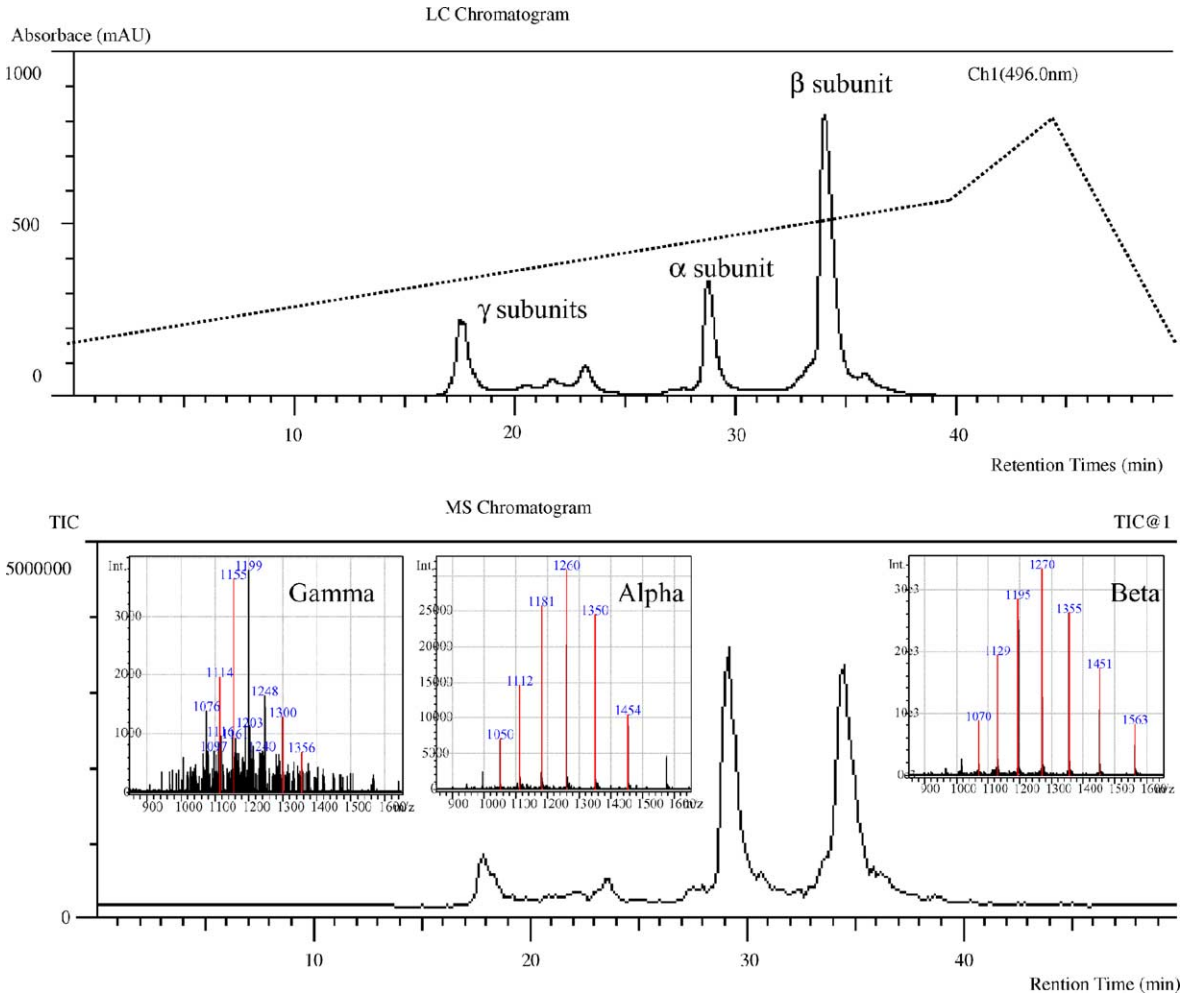

Fig. 9. HPLC–ESI-MS analysis of R-PE subunits. Dotted line represents the HPLC gradient in percentage of acetonitrile with 3.7% HCOOH (for gradient details see Section 2.8). Insets show the mass spectra of α, β and one of γ subunits (t_R = 17.5 min).

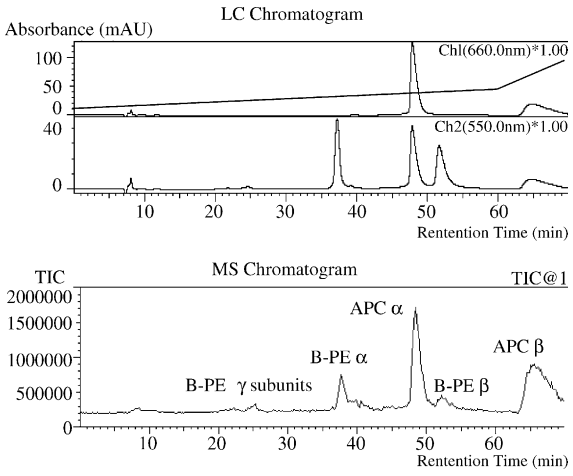

Fig. 10. HPLC–ESI-MS analysis of B-PE and APC subunits. Dotted line represents the HPLC gradient in percentage of acetonitrile with 3.7% HCOOH (for gradient details see Section 2.8).

3.6. HPLC–ESI-MS analysis of R-PE enzymatic digests

In the case of trypsin and pepsin digests, separations were efficient as well. Molecular weights of several PEB-containing peptides were determined (Fig. 12). Trypsin cuts amino-acid chains on the C terminus of lysine and arginine. Pepsin is relatively non-specific but preferentially cleaves the peptide bonds of hydrophobic amino acids. Triply charged ions found on peptides from trypsin digest originate from positive charges on the N terminus of the peptide, the N terminus of arginine, and the positively charged PEB chromophore. Double charged ions from pepsin digest originate from the positively charged peptide N terminus and the positively charged PEB chromophore. Sequences of several chromophore-containing peptides were deduced from sequences around the chromophore-binding sites in R-PE from *P. tenera* [7]. It is known that PEB is bound to cysteine resides 82 and 139 of the alpha subunit and to cysteine residues 82 and 158 of the beta subunit. PUB is bound to cysteines 50 and 61 of the beta subunit by two thioether bonds [7]. There is excellent agreement between

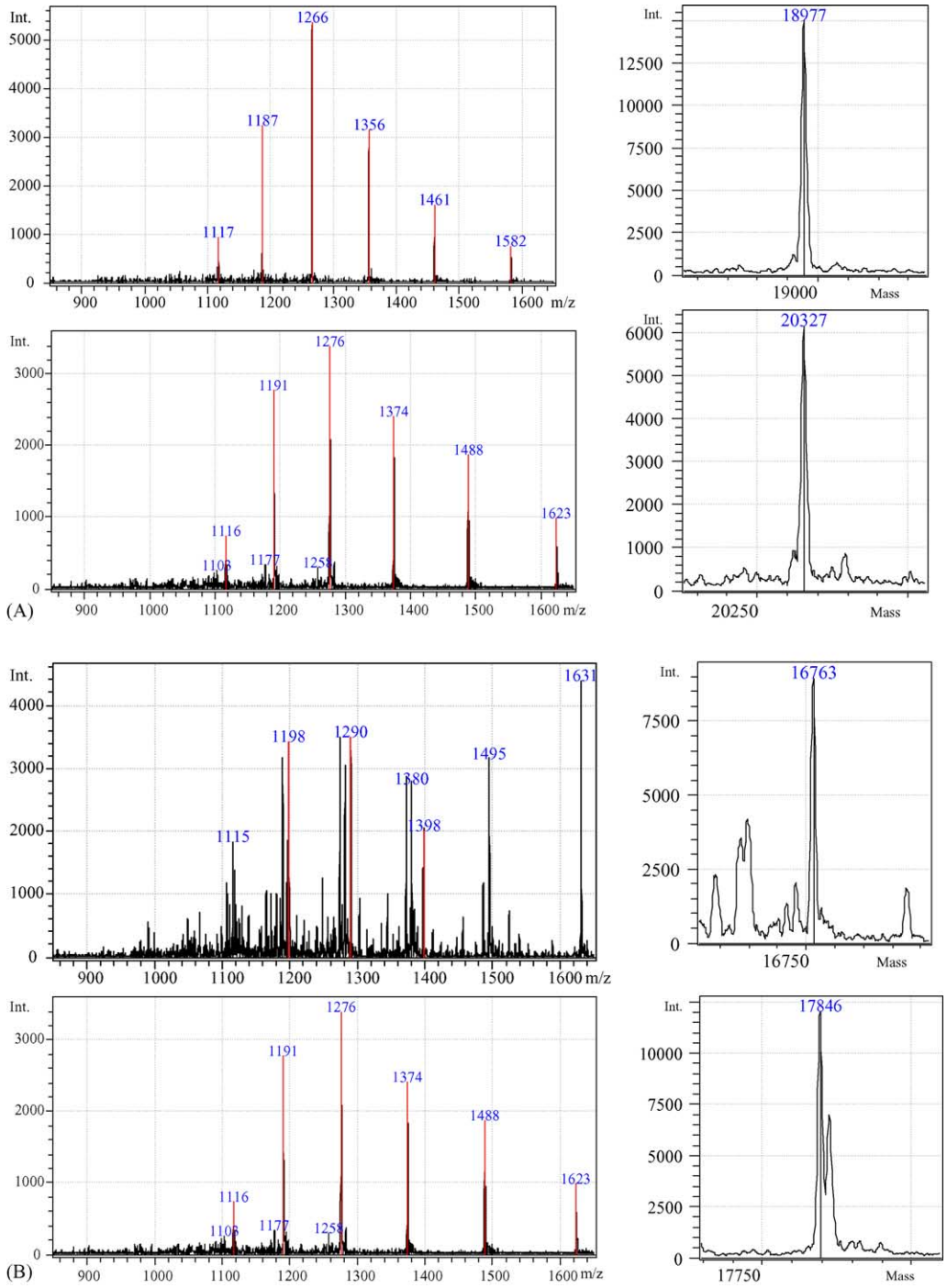

Fig. 11. (A) Mass spectra and molecular weights of B-PE α and β subunits. (B) Mass spectra and molecular weights of APC α and β subunits. M_r of one out of three determined values for APC α subunit is shown.

the measured peptide M_r values and peptide M_r values calculated from sequences of α and β subunits of R-PE from *P. tenera* (Table 4). There are several chromophore-containing peptides whose M_r values were not determined because they are larger than the *m*/*z* limit of the present MS quadrupole detector (2000 *m*/*z*). Sequences of these peptides could be found if MS–MS capabilities of the instrument are employed. That will make this method comparable in performance to amino acid sequencing of phycobilin-containing peptides [8].

3.7. Conclusion and future prospects

HPLC, CZE and SDS–PAGE separations followed by absorbance, fluorescence and MS detections were used for spectroscopic and structural characterization of R-PE

Table 4
Sequences of several PEB-containing peptides derived from HPLC–ESI-MS data and Swiss-Prot and TrEMBL protein database [7]

Digest	Retention time(min)	Measured molecular mass	Calculated molecular mass	Amino acid sequence
Pepsin digest	38.8	807.324	806.99	PEB-CV
	47.8	820.243	820.99	PEB-CL
	43.0	892.088	892.09	PEB-ACL
Trypsin digest	28.0	1027.322	1027.19	PEB-CYR
	34.8	1173.474	1173.49	PEB-LCVPR
	37.0	1250.406	1250.49	PEB-MAACLR

The molecular mass of PEB chromophore is 586.69. M_r values of peptides were calculated using program PAWS.

subunits and enzymatic digests. The same methodology could be employed in the analysis of other phycobiliproteins and fluorescent proteins in general. Along with their high absorbance, R-PE subunits and chromophore-containing peptides are highly fluorescent even under denaturing conditions and at the low pH conditions used in above separations. M_r values of R-PE subunits are smaller than the M_r of the widely used GFP while the values for their absorption coefficients are comparable. As shown here, one way to obtain these highly fluorescent molecules is semi-preparative chromatography. In vitro binding of chromophores for genetically expressed subunits [36] or solid-phase synthesized peptides are other pathways to obtain highly fluorescent R-PE peptides and subunits. It would be of the great interest to establish the biosynthesis pathway for these subunits, as was done for the alpha subunits of C-PC and PEC [37,38]. Phycobiliprotein subunits could be conjugated to different bioactive moieties as antibodies, histidine tags, streptavidin or biotin [39]. Although they are less bright than R-PE itself we show here that R-PE subunits can be detected down to the single-molecule level. They would interfere less with the system under interrogation (for example cells or surfaces) than R-PE because of the absence of interactions associated with quaternary structure and because of the smaller size compared to R-PE. Hence, R-PE subunits and chromophore-containing peptides have good potential for use as fluorescence probes in single-molecule detection and single-cell analysis.

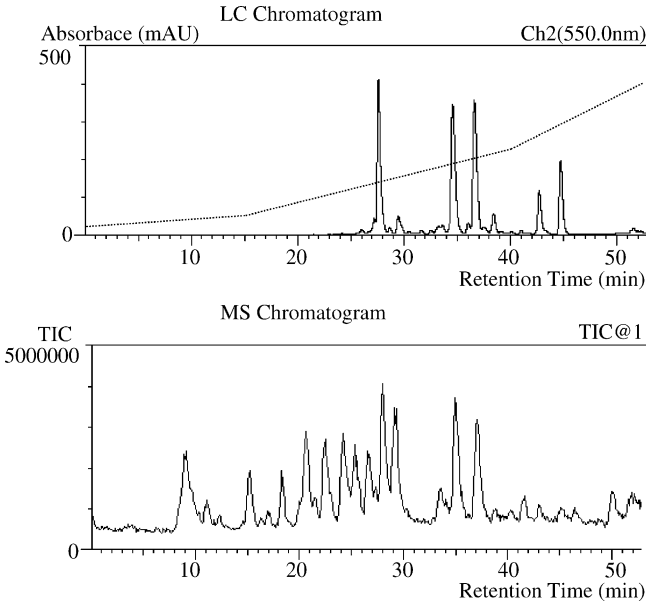

Fig. 12. HPLC–ESI-MS analysis of R-PE trypsin digest. Dotted line simbolizes the HPLC gradient in percentage of acetonitrile with 3.7% HCOOH (for gradient details see Section 2.8).

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