

# The Use of HPLC for the Purification of the Q<sub>B</sub>-Protein

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The Q<sub>B</sub>-protein is as a hydrophobic integral membrane protein firmly bound in the reaction center complex of photosystem II. A new method was developed to purify the SDS extracted protein using reversed-phase chromatography with two binary linear gradient systems. The identification of the Q<sub>B</sub>-protein was achieved by its rapidly labeling during photoassimilation of [<sup>35</sup>S]sulfate and by its reaction with the photoaffinity label azido-[<sup>14</sup>C]atrazine. Furthermore, antisera against the purified Q<sub>B</sub>-protein reacted with a single peak fraction, the second peak of the chromatogram, which was identical with the labeled protein peak fraction.

## Introduction

The Q<sub>B</sub>-protein of the photosystem II complex of *Chlamydomonas reinhardtii* can be easily labeled during the photoassimilation of [<sup>35</sup>S]sulfate in intact cells [1, 2]. Since many proteins have similar apparent molecular weights in the range of 32–35 kDa, it is of special interest that this protein can be labeled preferentially in light [1, 2] and identified by autoradiography.

The recent model of photosystem II locates the Q<sub>B</sub>-protein in the reaction center complex together with the Q<sub>A</sub>-protein [3]. Isolated reaction center complex preparations with the smallest possible photochemically active entity consist of both proteins and of cytochrome *b*<sub>559</sub> [4].

The integral Q<sub>B</sub>-protein with its extremely hydrophobic nature resists a number of common protein purification methods. Therefore, a new separation technique, HPLC-reverse phase chromatography, was applied for the purification of the Q<sub>B</sub>-protein.

## Materials and Methods

Mixotrophic cultures of *Chlamydomonas reinhardtii* were grown in presence of 15 mM acetate and 5% CO<sub>2</sub> in air as described earlier [5]. The cells were adjusted to 10 mg Chl/300 ml and preincubated for 60 min in light in presence of 30 mg cycloheximide. After the addition of [<sup>35</sup>S]sulfate (570 kBq/mg Chl) in

light (8 klx), the photoassimilation of sulfate was continued for 60 min and terminated by the addition of 30 ml cold 1 M sulfate.

### *The isolation of the rapidly labeled protein*

The cells were briefly sonicated and the resulting thylakoids were purified by gradient centrifugation and solubilized according to the method of Chua [6]. The proteins were separated on polyacrylamide gels with a linear gradient of acrylamide from 10 to 15% [6]. The Q<sub>B</sub>-protein was identified by autoradiography of the wet gel and the dominantly labeled band was excised. The protein was recovered by electroelution following the procedure of Hunkapiller [7]. The Q<sub>B</sub>-protein, which was not pure after this step, was further purified by a second electrophoresis in presence of 6 M urea.

### *Purification of the Q<sub>B</sub>-protein by HPLC-chromatography*

The Q<sub>B</sub>-protein contaminated with other proteins in the range of 32–35 kDa could be purified by reverse phase chromatography with consecutive linear gradients with different polarity. The chromatography was performed on a Vydac C 18 column (15 × 0.4) 218 TP 5415 with the binary system 0.1% TFA in H<sub>2</sub>O and 0.1% TFA in 84% acetonitrile. Hydrophilic proteins and contaminants from the polyacrylamide gel could be eluted while the Q<sub>B</sub>-protein was still bound to the column. A subsequent run with a linear gradient of 0.1% TFA in 60% HCOOH and 0.1% TFA in 60% HCOOH and 40% *n*-propa-

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nol eluted three protein peaks; of these the middle peak contained the Q<sub>B</sub>-protein.

#### *Amino acid analysis and determination of the N-terminus*

The three peak fractions were hydrolyzed for 24, 48 and 72 h in 6 N HCl and the amino acids in the hydrolysates were analysed as PTC-derivatives on a Spherisorb ODS II 3 µm column. The N-terminus of the unhydrolyzed Q<sub>B</sub>-protein was determined. The analysis was carried out on an Applied Biosystems model 470 A gas-phase-sequencer with an on-line PTH-Analyser.

#### *Photoaffinity labeling of the Q<sub>B</sub>-protein*

Purified thylakoids (equivalent to 5 mg Chl) were suspended in cold 20 mM tricine-NaOH buffer, pH 8.0, containing 20 mM MgCl<sub>2</sub>, and 50 nmoles of azido-[<sup>14</sup>C]atrazine were then added. The reaction was carried out in a quartz vessel, cooled in an icebath, under nitrogen atmosphere for 15 min in light (mercury lamp). The thylakoids were collected by centrifugation, dissolved in SDS and separated by electrophoresis as described above. The <sup>14</sup>C-labeled protein band was identified by autoradiography. The gel pieces were electroeluted and the protein concentrate was subjected to reverse phase chromatography as described above. Part of the gel pieces were oxidized with 30% hydrogenperoxide at 80 °C and the remaining dried particles prepared for liquid scintillation counting.

### **Results and Discussion**

*Chlamydomonas reinhardtii* cells were incubated with [<sup>35</sup>S]sulfate and the uptake reaction was completed – 98% of the original radioactivity – in 60 min. The incorporation of [<sup>35</sup>S] into the thylakoids corresponded to 15% of the total radioactivity, whereas only 0.5 to 1% of the label was recovered in the Q<sub>B</sub>-protein band. The recovery of the protein after the step of electroelution was nearly complete (85% of the original material).

The electroeluate was used for further purification by HPLC-reverse phase chromatography. The first gradient pair eluted mainly small contaminants from the polyacrylamide gel particles and unlabeled proteins. The change to the second gradient pair system resulted in the separation of three or four protein peaks as seen in Fig. 1. The first peak was phos-

phorylated in dark-light transition experiments with [<sup>32</sup>P]phosphate and intact cells, but not labeled with [<sup>35</sup>S]sulfate. This protein band was stained very well by coomassie blue and had an apparent molecular weight of 33 kDa, which is just below the Q<sub>B</sub>-protein band. Another phosphorylated protein band was above the Q<sub>B</sub>-protein, and recent experiments with an antibody against the Q<sub>A</sub>-protein made is possible to identify this protein (manuscript in preparation). The amino acid analysis of the first protein peak revealed that this protein is lysine rich.

The second protein peak, which was eluted from the column, had most of the [<sup>35</sup>S]radioactivity – 90% of the applied sample – and could be identified as the Q<sub>B</sub>-protein in a western blot with a monospecific antiserum against this protein. The latter protein was isolated by a different purification procedure without HPLC-chromatography.

The amino acid analysis of this peak showed only minute amounts of lysine. Additional evidence for

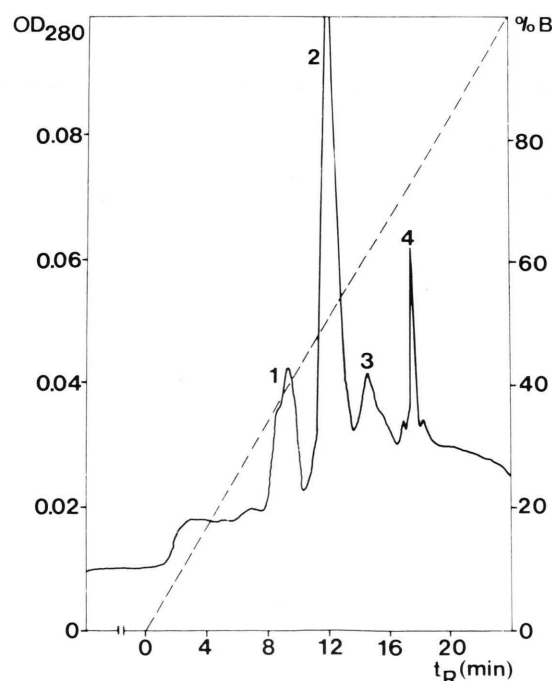


Fig. 1. HPLC-chromatogram of [<sup>32</sup>P] and [<sup>35</sup>S]labeled thylakoid proteins in the range of 32–35 kDa. The elution pattern was obtained with the second binary elution system (flow rate: 1 ml/min). After the gel electrophoresis the labeled protein bands were excised, electroeluted and applied to the HPLC-column. The phosphorylated protein was eluted in peak 1, whereas the [<sup>35</sup>S]labeled protein was eluted in peak 2. OD = optical density.

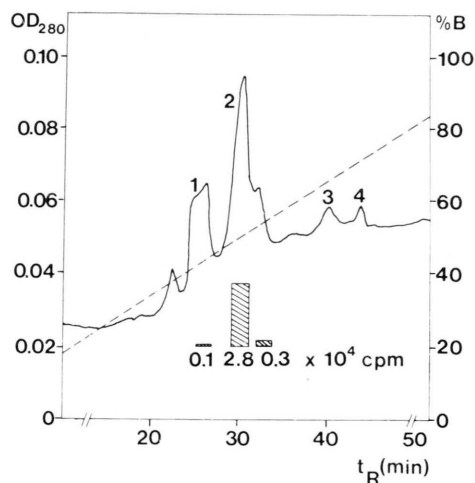


Fig. 2. HPLC-chromatogram of the azido-[<sup>14</sup>C]atrazine labeled Q<sub>B</sub>-protein, isolated from thylakoids, electroeluted and applied to the HPLC-column. The elution diagram shows the second elution system (flow rate: 1 ml/min). The radioactivity is indicated by the bar diagram. Peak 2 contains nearly the whole radioactivity. The comparison of the elution pattern with Fig. 1 shows that peak 2 contains the "rapidly labeled protein". OD = optical density.

the presence of the Q<sub>B</sub>-protein in this peak was achieved by the application of photoaffinity labeling technique with the HPLC-chromatography. Electroeluate of azido-[<sup>14</sup>C]atrazine labeled thylakoids enriched in the protein fraction of 32–35 kDa was separated by the same procedure as described above; the chromatogram is shown in Fig. 2. The determination of the distribution of the radioactivity revealed that 85% of the radioactivity applied to the column was recovered in the second peak at the same gradient composition as seen in Fig. 1. The purified Q<sub>B</sub> protein was used for the analysis of the N-terminus, but since this amino acid residue was blocked, the protein sequence could not be determined.

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