

S41-014

Identification of potential metal-binding proteins in the thylakoid lumen of spinach

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Keywords: thylakoid lumen, affinity chromatography, spinach, copper

Introduction

Within the chloroplast the thylakoid membrane encloses the lumen compartment, which still is a quite unexplored area of the chloroplast. Using a proteomic approach the number of proteins in the lumen compartment is estimated to be approximately 70 (Schubert, M., et al., 2001). The copper binding plastocyanin and the three extrinsic proteins PsbO, PsbP and PsbQ, that are associated with the manganese cluster of Photosystem II, are probably the best known and most abundant proteins in the thylakoid lumen. Several luminal proteins are known to bind various cofactors, such as metals, for their enzymatic functions. Recently a number of proteins have been identified (Kieselbach, T. et al., 1998, Mant, A. et al., 1999 and Schubert, M., et al., 2001), but for many of them the structure and function is still unknown.

In this work we have approached the problem of identifying possible candidates for metal binding in the lumen of spinach, by using Immobilised Metal Affinity Chromatography (IMAC) that separates peptides and proteins according to their affinity for immobilised metal ions. Isolated and purified lumen was applied to a HiTrap column loaded with Cu²⁺, which gives a strong ligand-protein interaction.

Materials and methods

Preparation of the lumen fraction - The luminal content was isolated from spinach (*Spinacia oleracea*) according to Kieselbach et al. (1998). The protein concentration was measured according to Bradford (1976) with bovine serum albumine as a standard. The chlorophyll concentration was determined as in Porra et al. (1989) after extraction in 80% acetone.

Chromatography techniques - Between 5-10 mL lumen fraction (300-500 µg protein/mL) were spin dialysed with Centricon YM-3 (Millipore) with buffer A (20 mM Sodium phosphate, 0,5 M NaCl, pH 6.0) to adjust the salt concentration and the pH of the sample. This was followed by a centrifugation in a microfuge (13000 rpm for 30 min) to pellet aggregations of insoluble proteins and lipids. This treatment gave a highly pure lumen fraction with a final volume of about 1.5 mL (1-2 mg protein/mL) that was applied onto the column.

To isolate copper binding proteins an ÄKTAexplorer system was used to which a HiTrap Chelating column, 5 ml (both from Amersham Pharmacia Biotech) was connected. The column was charged with Cu^{2+} , according to the column manual. The absorbance was continuously measured at 280 nm. Unbound proteins were washed out directly followed by a 0.6 column volume of 0.1% Triton X-100. When the absorbance decreased and was stable close to zero, a step-wise elution was performed with a decrease in pH of buffer A, from pH 5.0 down to pH 3.0.

Electrophoretic analysis - 1D SDS-PAGE was performed with 18% polyacrylamide (w/v), 2 M urea slab gels, by the method described in Leammli (1970). For the 2D-gel electrophoresis the fractions of interest were spin-dialysed with Centriprep YM-3 (Millipore) with water to obtain a salt concentration less than 10 mM. 2D-electrophoresis and image analysis was performed as in Kieselbach et al. (2000). The gels were silver stained as in Bjellqvist et al. (1993).

Results

Fig. 1 shows a typical elution profile for the isolation of metal binding proteins from the thylakoid lumen. The experiment was repeated several times using different preparations of lumen extractions. Each time only a small volume of the sample was used to prevent weakly bound proteins to elute during application of the sample. The first peak of the elution profile contained the void volume. About 0.6 column volumes of 0.1% Triton X-100 were added during elution to prevent hydrophobic interactions between the sample and the gel matrix (second elution peak, Fig. 1). The column was then washed with buffer A until the absorbance was stable at around zero. A step gradient was used by lowering the pH from 5 until 3 to elute the bound proteins from the column. The fractions corresponding to the different peaks eluted at pH 5, 4 and 3 were then collected for further study.

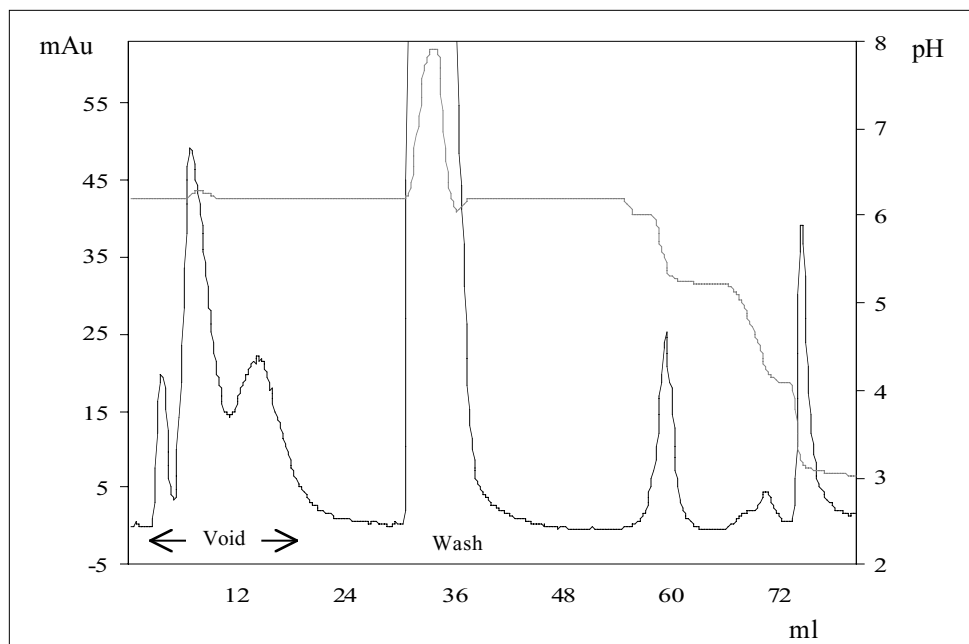


Fig. 1. Elution profile showing the absorbance measured at 280 nm on the left axis and the pH changes on the right axis.

Figure 2 shows the eluted proteins analysed by SDS PAGE. For comparison the total lumen content were loaded in lane 1. Lane 2, 3 and 4 shows the proteins eluted at pH5, pH 4 and pH 3, respectively. PsbQ is one of the major proteins, that is eluted at pH5, but it is present in all fractions. It seems to have a high affinity for copper, which is not unexpected considering that it has been proposed to be involved in binding Ca^{2+} . Both the pH4 and pH3 fractions contain Ferredoxin-NADP-reductase (FENR), a known stromal thylakoid associated protein that easily contaminates the lumen fraction during isolation. FENR binds Fe^{2+} and thus serves as a positive control as it binds to the column.

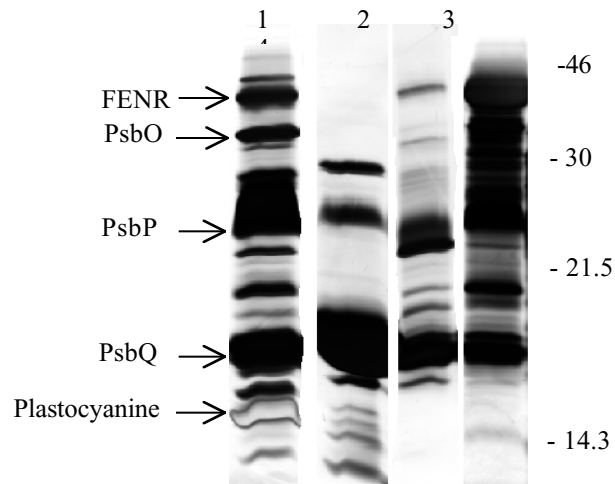


Fig. 2, In lane 1 is lumen, Lane 2, pH 5, Lane 3, pH 4 and in Lane 4, pH 3.

The total yield of each eluted peak was further analysed by 2D-PAGE. Gel matching was made comparing three different pH4 gels showed high similarity. This revealed at least 10 putative metal binding proteins to conduct further study on. So far three of these have been identified as FENR, PsbQ and an unknown 24.8 kDa protein that are marked in fig. 3. Further identification are in progress.

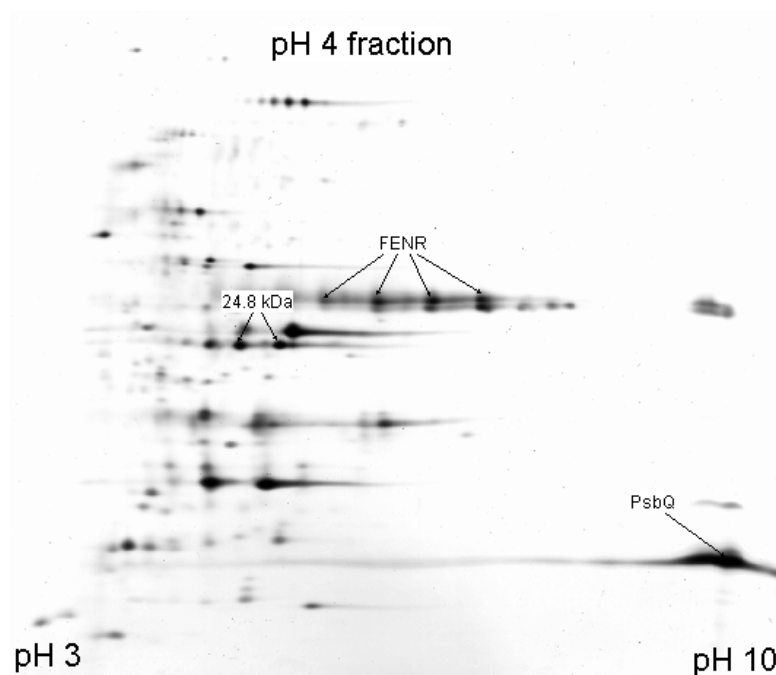


Fig. 3, 2D map of the pH 4 peak.

Discussion

A 2D-gel analysis of the thylakoid lumen of *A. thaliana* shows the presence of about 70 proteins (Schubert, M. et al., 2001). In this work we wanted to investigate which of these proteins could be involved in metal binding. Metal cofactors that are present and utilised in the lumen are Mn, Ca^{2+} , Fe^{2+} and Cl^- in PSII, Fe^{2+} in the cytochrome b_6/f complex and peroxidases located in the lumen (Kieselbach et al., 2000) and Cu^{2+} in plastocyanine and polyphenoloxidase.

When analysing the elution peaks on 1D-PAGE several protein bands were detected at pH 5 -3, including some of the dominating lumen proteins, PsbO, PsbP, and PsbQ. As seen in Fig. 1, a pH of 3 was needed to elute the strongest bound proteins, which thus must have a very high affinity for copper ions, since at this pH the proteins are totally denatured.

Since proteomics is an excellent method for further study of the low abundance proteins, the metal binding column fractions were analysed by 2D-gel electrophoresis. As seen in Fig 3, several interesting protein spots were detected as possible candidates for metal binding. Further analyses of these potential metal binding proteins will be performed using N-terminal Edman sequencing and MALDI-TOF MS.

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