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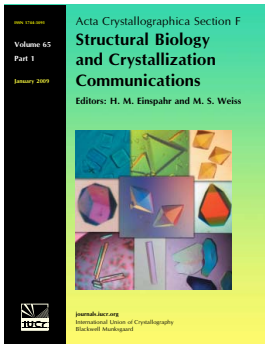

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Crystallization and preliminary crystallographic characterization of the extrinsic PsbP protein of photosystem II from *Spinacia oleracea*

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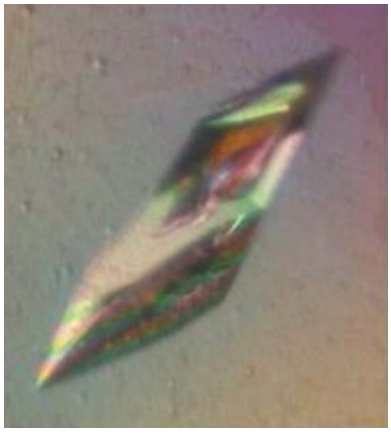


Preliminary X-ray diffraction analysis of the extrinsic PsbP protein of photosystem II from spinach (*Spinacia oleracea*) was performed using N-terminally His-tagged recombinant PsbP protein overexpressed in *Escherichia coli*. Recombinant PsbP protein (thrombin-digested recombinant His-tagged PsbP) stored in bis-Tris buffer pH 6.00 was crystallized using the sitting-drop vapour-diffusion technique with PEG 550 MME as a precipitant and zinc sulfate as an additive. SDS–PAGE analysis of a dissolved crystal showed that the crystals did not contain the degradation products of recombinant PsbP protein. PsbP crystals diffracted to 2.06 Å resolution in space group $P2_12_12_1$, with unit-cell parameters a = 38.68, b = 46.73, c = 88.9 Å.

1. Introduction

Oxygenic photosynthesis is the process by which light energy is converted into chemical energy. This process takes place in the thylakoid membrane of higher plants, algae and cyanobacteria, in which the membrane-embedded pigment–protein complex photosystem II (PSII) performs light-driven oxidation of water with concomitant reduction of the plastoquinone pool. As a result of the light-driven redox reaction, molecular oxygen is released as a byproduct from the oxygen-evolving complex (OEC) located on the lumenal side of PSII (Barber, 2003). The extrinsic proteins stabilizing the OEC catalytic centre differ in prokaryotic and eukaryotic oxyphototrophs (Seidler, 1996; De Las Rivas *et al.*, 2004). The OEC of higher plants consists of an inorganic Mn_4Ca cluster and extrinsic proteins named PsbO, PsbP, PsbQ and PsbR, which create the correct ionic environment during water oxidation. The PSII of nongreen algae and cyanobacteria also includes PsbO, but two different extrinsic proteins, PsbU and PsbV, are associated with the OEC catalytic centre (Seidler, 1996; Suorsa *et al.*, 2006). For a better understanding of the water splitting, knowledge of the structure of PSII and its extrinsic proteins is an essential prerequisite. The recently determined three-dimensional X-ray structure of a cyanobacterial PSII has notably improved upon the previous partial structures of the bacterial complex (Zouni *et al.*, 2001; Kamiya & Shen, 2003; Loll *et al.*, 2005). However, these cyanobacterial PSII structures provide no clues to the possible arrangement of the extrinsic PsbP and PsbQ proteins on the lumenal side of higher plant PSII. While PsbO is ubiquitous to all known oxyphototrophs, PsbP and PsbQ are not. Moreover, PsbU and PsbV are not homologous in sequence or structure to PsbP or PsbQ, although they play the same functions as the PsbP and PsbQ proteins of the higher plant and green algal PSII (Shen & Inoue, 1993). *In vitro* studies have demonstrated that PsbP and PsbQ are involved in Ca^{2+} and Cl^- retention in PSII and also form a barrier that is open to substrates and products but closed to exogenous reductants (Seidler, 1996). More recently, the physiological roles of PsbP and PsbQ have been tested in transgenic plants in which the levels of these two proteins were severely downregulated. Whereas PsbP was demonstrated to be essential for the regulation and stability of PSII (Ifuku, Yamamoto *et al.*, 2005; Yi *et al.*, 2007), PsbQ was found to be dispensable (Ifuku, Yamamoto *et al.*, 2005). In recent years, efforts to

elucidate the X-ray structures of PsbP and PsbQ have been made in order to shed further light on their role in the OEC of green plant PSII. The crystal structure of PsbQ from *Spinacia oleracea* has been solved to 1.49 Å resolution (Balsera *et al.*, 2005). To date, recombinant PsbP proteins from several plant species (spinach, cucumber and tobacco) have been tested for crystallization, but only the crystal structure of PsbP protein from *Nicotiana tabacum* has been solved (to 1.60 Å resolution; Ifuku *et al.*, 2004). Partial degradation at the N-terminus of the protein was observed in these PsbP crystals (Ifuku *et al.*, 2003). However, the region prone to degradation is reported to be functionally relevant (Ifuku, Nakatsu *et al.*, 2005). Here, we report the successful purification and crystallization of recombinant PsbP protein from *S. oleracea* without partial degradation.

2. Materials and methods

2.1. Isolation of spinach PsbP protein overexpressed in *Escherichia coli* with a His anchor

Coding regions for the mature PsbP protein were amplified by high-fidelity PCR using the clone pSoc23.81E1 as a template (Jansen *et al.*, 1987). This clone was a kind gift from Professor R. G. Herrmann. The PCR primers used were 5′-C**CAT*ATG***GCCTATGGAGAAGCTGCTAAT-3′ (forward) and 5′-G**GGATCC***TTA*AGCAACACTGAAAGAACT-3′ (reverse) containing restriction sites for *Nde*I and *Bam*HI (bold nucleotides) and a start codon and a termination anticodon (italicized nucleotides), respectively. The resulting PCR product was purified using the GFX PCR DNA and gel band purification kit (GE Healthcare Biosciences AB, Uppsala, Sweden) and cloned into *Eco*RV-digested Bluescript II SK$^+$ vector (BSK$^+$). The PCR-amplified *psbP* insert was sequenced in both directions in an automated sequencer using the reverse and universal primers to verify the nucleotide sequence. The plasmid DNA containing the *psbP* insert was consecutively cut with *Nde*I and *Bam*HI and cloned into pET-12a (Unigene) pre-digested with *Nde*I and *Bam*HI. The resulting construction was named JR2591. The chosen forward primer including an *Nde*I restriction site introduced a methionine residue in the first N-terminal position of the recombinant PsbP protein. JR2591 was transferred into *E. coli* BL21(DE3)pLysS. The region of the upstream leader *ompT* of pET12a, encoding the outer membrane protein specified by *ompT*, was removed when using the restriction enzymes *Nde*I and *Bam*HI. Transformed cells named B95 were stored at 193 K in a 20% glycerol solution. The PsbP expression resulting from JR2591 was minimal regardless of temperature or the optical density of the cell culture after IPTG induction. As a consequence of the poor expression, the pET-28b+ vector (Novagen) was chosen as an alternative expression system. BSK$^+$ containing the *psbP* insert was subsequently cut with *Nde*I and *Bam*HI as described above and cloned into a pET-28b+ vector pre-digested with *Nde*I and *Bam*HI. The resulting construction was named JR3133. In this case, the recombinant PsbP protein contained a His-tagged sequence and a thrombin cleavage site in the N-terminal region. JR3133 was transferred into *E. coli* BL21(DE3)pLysS. Transformed cells named B152 were stored at 193 K in a 20% glycerol solution.

2.2. Purification of recombinant PsbP and His-tagged PsbP proteins

B152 cells containing the JR3133 construct were grown at 310 K in LB medium supplemented with 50 µg ml^{-1} kanamycin. When the optical density of the culture at 580 nm reached a value of 0.6, overexpression of the His-tagged recombinant PsbP protein was initiated by adding 1 m*M* IPTG. The cells were incubated for 18 h at 303 K and then harvested by centrifugation, suspended in 20 m*M* potassium phosphate, 1 m*M* EDTA, 0.5 *M* NaCl pH 7.4 (buffer *A*) and finally passed through a French press. After removing the unbroken material by centrifugation at 15 000*g* for 45 min at 277 K, the protein suspension was loaded onto an Ni Sepharose High Performance column (GE Healthcare Biosciences AB, Uppsala, Sweden) pre-equilibrated with buffer *A*. The His-tagged recombinant PsbP protein was eluted with a linear gradient of increasing imidazole concentration from 0 to 0.5 *M* in 20 m*M* potassium phosphate, 1 m*M* EDTA, 0.5 *M* NaCl, 0.5 *M* imidazole pH 7.4 (buffer *B*). Fractions enriched in the His-tagged recombinant PsbP protein were pooled, concentrated and washed with 20 m*M* bis-Tris, 1 m*M* EDTA pH 6.0 (buffer *C*) using centrifugal filter devices (Amicon Ultra 10 000 molecular-weight cutoff, 15 ml capacity) from Millipore (Billerica, Massachusetts, USA). The His-tagged recombinant PsbP protein was loaded onto an SP Sepharose Fast Flow cation-exchange column (GE Healthcare Biosciences AB, Uppsala, Sweden) pre-equilibrated with buffer *C* and eluted with a linear gradient of increasing salt concentration from 0 to 0.7 *M* in 20 m*M* bis-Tris, 1 m*M* EDTA, 1 *M* NaCl pH 6.0 (buffer *D*). Fractions enriched in the His-tagged recombinant PsbP protein were pooled, washed with 20 m*M* bis-Tris, 0.1 *M* NaCl pH 6.0 (buffer *E*) and finally concentrated to 15 mg ml^{-1} using centrifugal filter devices (Amicon Ultra 10 000 molecular-weight cutoff, 15 ml capacity) from Millipore (Billerica, Massachusetts, USA). The first 17 amino acids of the N-terminal region of the His-tagged recombinant PsbP protein were enzymatically removed with thrombin (Calbiochem, EMD Biosciences Inc., San Diego, California, USA) by incubating 5 mg of protein per unit of protease in buffer *E* for 3 h at room temperature. As the result of protease cleavage, the His-tagged sequence was removed and the resulting recombinant PsbP protein contained four additional amino acids (*i.e.* GSHM) at the N-terminus compared with the native PsbP protein from spinach. The recombinant PsbP protein was passed through a Superdex 75 column (GE Healthcare Biosciences AB, Uppsala, Sweden) pre-equilibrated with 20 m*M* bis-Tris, 1 m*M* EDTA, 0.2 *M* NaCl pH 6.0 (buffer *F*) at a flow rate of 0.2 ml min^{-1}. Fractions containing the recombinant PsbP protein were collected and concentrated to 15 mg ml^{-1} using centrifugal filter devices (Amicon Ultra 10 000 molecular-weight cutoff, 4 ml capacity) from Millipore (Billerica, Massachusetts, USA). SDS–PAGE according to Laemmli (1970) with a total acrylamide content of 12% in the separating gel was performed to test the purity of the recombinant PsbP protein. The gel was stained with Coomassie R-250.

2.3. Protein crystallization and data collection

The recombinant PsbP protein was washed with 20 m*M* bis-Tris pH 6.0 and concentrated to a final concentration of about 15 mg ml^{-1} using centrifugal filter devices (Amicon Ultra 10 000 molecular-weight cutoff, 4 ml capacity) from Millipore (Billerica, Massachusetts, USA). The protein was crystallized at room temperature using the sitting-drop vapour-diffusion technique. The Crystallization Basic Kit for Proteins and the Crystallization Extension Kit for Proteins (Sigma) were used to find initial crystallization conditions. A 2 µl volume of protein solution (15 mg ml^{-1}) was mixed with 2 µl of various reservoir solutions and equilibrated against 700 µl reservoir solution. Screening showed that PEG 550 MME, PEG 6000 and PEG 8000 were effective precipitants. Univalent and bivalent ions were tested as additives in subsequent experiments. The conditions were optimized by varying the protein concentration, additives and buffer systems (type, concentration and pH).

Crystals were tested using the in-house X-ray diffractometer of the Institute of Organic Chemistry and Biochemistry and the Institute of

Molecular Genetics of the Academy of the Sciences of the Czech Republic in Prague. Protein crystals were measured and complete data sets were collected using an in-house X-ray diffractometer constituted of an FR591 rotating-anode X-ray generator (Bruker–Nonius), a double-mirror X-ray optical system (XOS), a MAR 345 image-plate detector and a Cryostream cooling system (Oxford Cryosystem) using Cu $K\alpha$ radiation. Crystals were cryocooled in a nitrogen-gas stream at 110 K. A total of 231° of diffraction images were collected, each covering 0.5° crystal rotation. The measured data were integrated using *MOSFLM* (Leslie, 1999) and scaled using *SCALA* (Evans, 1997), both from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Successful purification and crystallization of the recombinant PsbP protein from *S. oleracea* is reported in this paper. Protein purification began with overexpression of the recombinant PsbP protein with a six-His tag at the N-terminus (His-tagged PsbP protein), which was followed by a series of chromatographic steps including the application of metal-affinity and ion-exchange chromatography. Size-exclusion chromatography was added as the final step of the protein-purification procedure to separate the recombinant PsbP protein from the protease and the cleaved His tag (Fig. 1). A test of the stability of the recombinant His-tagged PsbP and recombinant PsbP proteins in different buffers (0.1 *M* MES pH 6.0, 20 m*M* bis-Tris pH 6.0, 0.1 *M* sodium cacodylate buffer pH 6.5 and 20 m*M* potassium phosphate buffer pH 7.4) showed that they displayed highest stability in 20 m*M* bis-Tris buffer pH 6.0 (data not shown). Two different buffers (20 m*M* bis-Tris buffer, 0.1 *M* NaCl pH 6.00 and 20 m*M* potassium phosphate buffer, 0.1 *M* NaCl pH 7.4) were tested and compared for thrombin activity. No differences in the cleavage of the His anchor by thrombin were observed (data not shown). Bis-Tris buffer was found to be an optimal buffer for all the purification steps that followed purification of the His-tagged PsbP protein and also for crystallization experiments.

Both recombinant proteins (His-tagged PsbP and PsbP) were used for crystallization trials. However, only recombinant PsbP protein was found to form three-dimensional crystals, which were observed

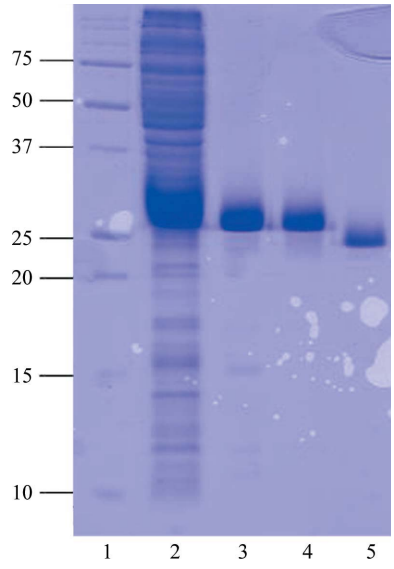
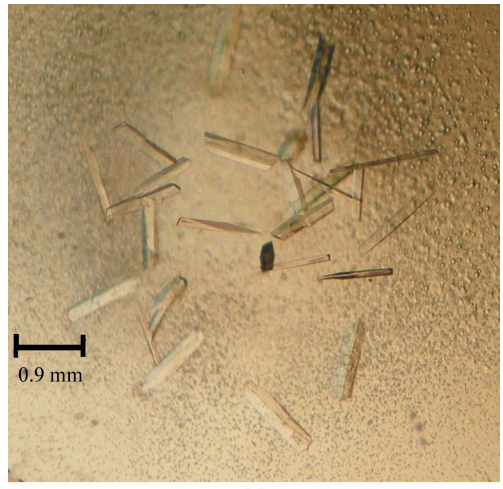


Figure 1
SDS–PAGE analysis of the purification steps of the spinach recombinant PsbP protein. Lane 1, molecular-weight markers (kDa); lane 2, supernatant after IPTG induction of B152 cells; lane 3, the His-tagged PsbP protein purified by metal-affinity chromatography; lane 4, His-tagged PsbP protein purified by ion-exchange chromatography; lane 5, recombinant PsbP protein after Superdex 75 column chromatography.



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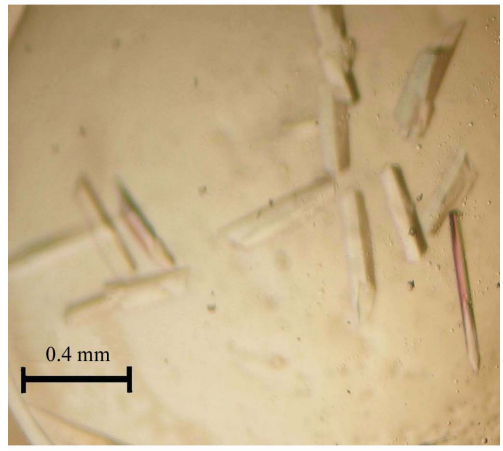

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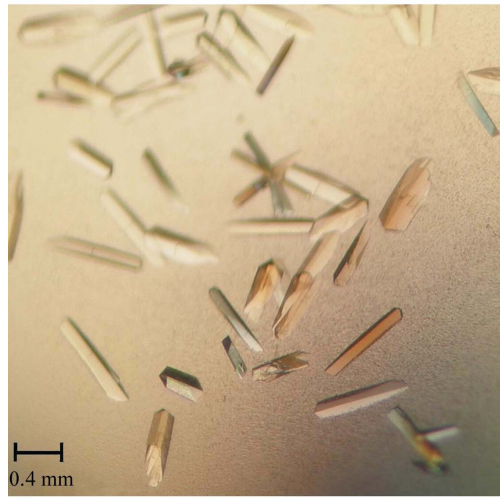

(*c*)

Figure 2
PsbP crystals from three different conditions: (*a*) 13% PEG 8000, 0.1 *M* Tris–HCl pH 7.5, 10 m*M* $ZnCl_2$, (*b*) 18% PEG 6000, 20 m*M* zinc acetate, 0.1 *M* Tris–HCl pH 7.0 and (*c*) 16% PEG 550 MME, 0.1 *M* Tris–HCl pH 7.5, 10 m*M* $ZnSO_4$.

Table 1
Data-collection statistics for the PsbP crystal.

Values in parentheses correspond to the highest resolution shell.

X-ray source	Home source
Wavelength (Å)	1.54179
Temperature (K)	110.0
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 38.72, b = 46.79, c = 89.01
Resolution limits (Å)	29.83–2.06 (2.18–2.06)
R_{merge}†	0.045 (0.111)
No. of unique reflections	10235 (1258)
Completeness	97.6 (80.3)
Multiplicity	4.4 (4.3)
$\langle I/\sigma(I)\rangle$	24.6 (10.8)
Wilson B (Å^2)	23.2

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

under three different conditions (Fig. 2). Optimization of the crystallization conditions showed that the following condition yielded the best quality crystals: 15 mg ml^{-1} PsbP protein in 20 m*M* bis-Tris buffer pH 6.0 mixed in a 1:1 ratio with reservoir solution containing 20% PEG 550 MME, 0.1 *M* Tris–HCl pH 7.5 and 5 m*M* $ZnSO_4$ and equilibrated at 288 K for 3 d (Fig. 3). SDS–PAGE analysis of the dissolved crystals showed that crystals were only formed from mature protein; no partial degradation of PsbP was observed (Fig. 4). Formation of PsbP crystals under the described conditions was found to be advantageous in comparison with previously published crystallization conditions (Ifuku *et al.*, 2003), which yielded PsbP crystals that contained partially degraded protein. Apart from the concrete crystallization conditions, the shorter time needed for crystal growth (3 d for PsbP protein from *S. oleracea* compared with two weeks for PsbP protein from *N. tabacum*) might play a role in preventing degradation. Diffraction data were collected to 2.06 Å resolution from a protein crystal directly frozen in mother liquor at 110 K (Fig. 3). Crystal parameters and data-collection statistics are summarized in Table 1. Our attempts to solve the phase problem by molecular replacement were successful when the structure of the PsbP protein from *N. tabacum* (PDB code 1v2b; Ifuku *et al.*, 2004) was used as a search model. The R factor and R_{free} factor were 0.453 and 0.463, respectively, and fell to 0.446 and 0.458, respectively, during the initial three cycles of rigid-body refinement for data in the resolution range 44.5–3.8 Å. The orthorhombic crystal form (space group $P2_12_12_1$) contained one molecule in the asymmetric unit, with a solvent content of 36.2% (V_M = 1.92 Å^3 Da^{-1}).

4. Conclusion

The expression, purification and crystallization of stable recombinant forms of the extrinsic PsbP protein of PSII are essential steps in the elucidation of its three-dimensional structure and its interaction with other PSII subunits. Knowledge of the crystallographic structures of PsbP from tobacco (Ifuku *et al.*, 2004) and spinach will allow us to relate their structures to the data available from biophysical experiments (Raman spectroscopy, FTIR spectroscopy and molecular dynamics). Likewise, the structural information obtained on this PSII extrinsic protein will lead to the design of further experiments (vibrational spectroscopy, NMR, protein–protein cross-linking, surface plasmon resonance and atomic force microscopy) in order to

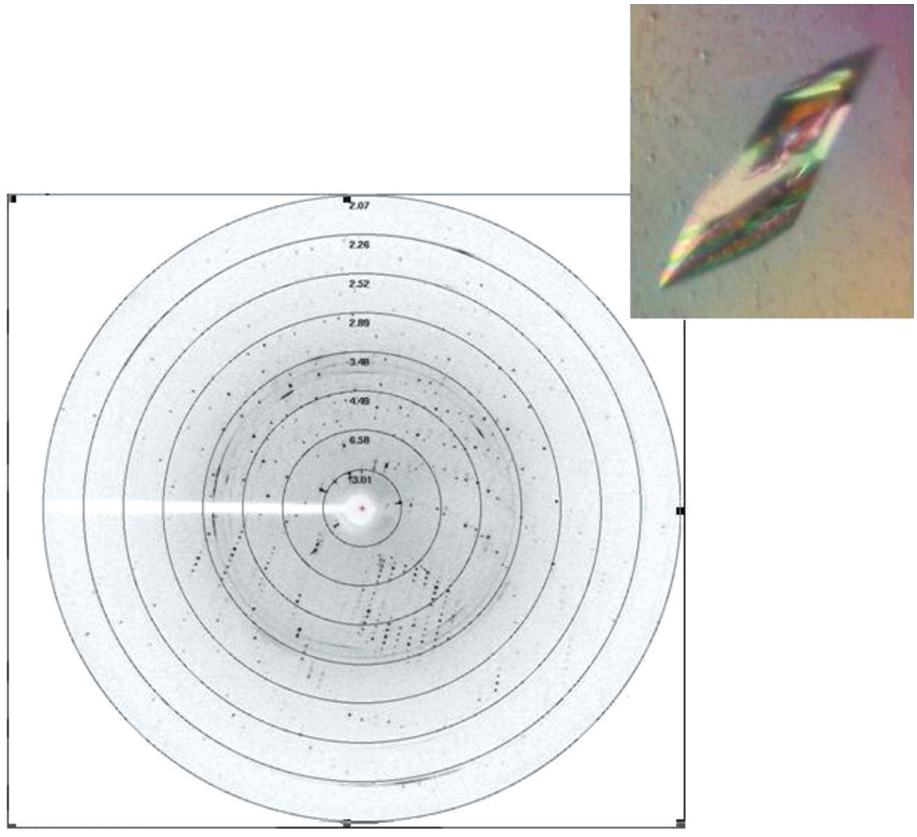

Figure 3
Difraction image of the spinach recombinant PsbP protein crystallized using 20% PEG 550 MME, 0.1 *M* Tris–HCl pH 7.5 and 5 m*M* $ZnSO_4$.

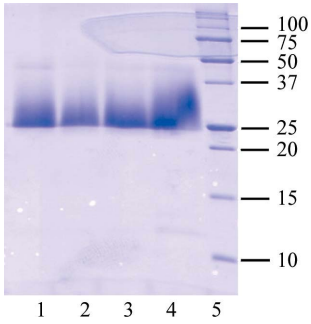

Figure 4
SDS–PAGE analysis of dissolved PsbP crystals obtained from four different conditions: lane 1, 20% PEG 550 MME, 0.1 *M* Tris–HCl pH 7.0, 20 m*M* $ZnCl_2$; lane 2, 15% PEG 550 MME, 0.1 *M* Tris–HCl pH 7.0, 20 m*M* $ZnSO_4$; lane 3, 15% PEG 550 MME, 0.1 *M* Tris–HCl pH 7.5, 20 m*M* $ZnSO_4$; lane 4, 15% PEG 8000, 0.1 *M* Tris–HCl pH 7.5, 20 m*M* $ZnCl_2$; lane 5, molecular-weight markers (kDa).

better understand the role of extrinsic proteins in PSII of higher plants.

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