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# Detection of calcium binding by photosystem II polypeptides immobilised onto nitrocellulose membrane

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Photosystem II calcium-binding polypeptides have been detected by their ability to selectively bind $^{45}Ca$ when immobilised onto nitrocellulose membrane following SDS-polyacrylamide gel electrophoresis. Two calcium-binding polypeptides of 26 kDa and 24 kDa are shown to be components of LHCII. The 24 kDa polypeptide was further characterised by N-terminal amino acid sequence analysis and shown to be the product of a type II *cab* gene. A third polypeptide of 33 kDa bound calcium more weakly and was not positively identified.

Calcium-binding polypeptide; Light-harvesting complex II; Photosystem II; N-terminal sequence

## 1. INTRODUCTION

Calcium is an essential co-factor for photosystem II reactions [1–4]. The calcium ions are required for the normal functioning of the water-oxidising complex and for electron flow from Z to $P680^+$ [2–4]. The minimum number of calcium ions required for photosystem II activity has been reported to range between 1 and 3 per photosystem II reaction center [5,6], and may depend on the species of plant used [5]. Although the nature and location of the calcium-binding sites is unknown, much of the evidence points to the involvement of proteins. Reports that exogenous calcium can partially reverse the inhibition of photosynthetic oxygen evolution resulting from depletion of the extrinsic 33, 23 and 16 kDa polypeptides by salt washings [3,7–9] has led to proposals that an extrinsic polypeptide provides a high-affinity binding site for calcium. However, observations that light is required for the release of calcium from salt-washed photosystem II membranes indicates an intrinsic polypeptide as the calcium-binding site [10]. Maruyama et al. [11] have shown that calcium-binding proteins are able to specifically bind $^{45}Ca$ when immobilised onto nitrocellulose membrane following SDS-polyacrylamide gel electrophoresis. This technique has also been successfully used to detect a calcium-binding protein in *Streptomyces erythreus* [12]. In this paper we have used this method to detect individual polypeptides of photosystem II that are able to bind $^{45}Ca$ selectively when immobilised onto nitrocellulose membrane.

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*Abbreviations:* DCCD, *N,N'*-dicyclohexylcarbodiimide; LHC, light-harvesting complex; OGP, 1-*O*-*n*-octyl-β-D-glucopyranoside; PVDF, polyvinylidene difluoride

## 2. MATERIALS AND METHODS

Photosystem II membranes were prepared by solubilising washed wheat thylakoid membranes with Triton X-100 at a detergent to chlorophyll ratio of 25:1 (w/w) following the procedure of Berthold et al. [13]. Photosystem II membranes were further solubilised with 35 mM OGP in high salt buffers to isolate LHCII and photosystem II-core complexes as described by Ghanotakis and Yocum [14]. Photosystem II and LHCII polypeptides were solubilised in SDS sample buffer (2% SDS, 10 mM mercaptoethanol, 0.1 M Tris-HCl, pH 6.8, and 30% glycerol) and fractionated by SDS-polyacrylamide gel electrophoresis using a 15% acrylamide resolving gel and the buffers of Laemmli [15]. When used DCCD was incubated with

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membrane preparations using 2 $\mu$M DCCD to 1 $\mu$M chlorophyll [16].

Polypeptides able to bind $^{45}Ca$ were detected using the method essentially reported by Maruyama et al. [11]. Following electrophoresis polypeptides were transferred to nitrocellulose membrane using the method of Towbin et al. [17]. Transfer was performed at a constant 100 mA for 30 min in transfer buffer containing 20% methanol, 0.025 M Tris and 0.129 M glycine, pH 8.5. After transfer the nitrocellulose membrane was washed three times with a solution containing 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole-HCl (pH 6.8) for a total time of 1 h. The membrane was then incubated for 10 min with 10 mM imidazole-HCl (pH 6.8), 60 mM KCl, 5 mM $MgCl_2$ and 30 $\mu$Ci $^{45}CaCl_2$ (20 mCi/mg $Ca^{2+}$) and subsequently washed with 50% ethanol for 5 min. Polypeptides able to retain $^{45}Ca$ after washing were visualised by autoradiography.

Polypeptides to be sequenced were transferred to PVDF membrane [18] and submitted for sequencing on an Applied Biosystems 477A pulsed-liquid phase protein sequencer with an on-line 120A phenylthiohydantoin amino acid analyser using standard NORMAL-1 software.

## 3. RESULTS AND DISCUSSION

Photosystem II polypeptides fractionated by SDS-polyacrylamide gel electrophoresis were transferred onto nitrocellulose membrane and incubated with $^{45}CaCl_2$ [11]. Following subsequent washings of the nitrocellulose filter with 50% ethanol, polypeptides able to retain $^{45}Ca$ were visualised by autoradiography (fig.1). The usefulness of this method to detect calcium-binding polypeptides is demonstrated in fig.1 (lane 1) by the ability to observe uptake and retention of $^{45}Ca$ by bovine calmodulin. This method has been shown to be useful for detecting a range of mammalian calcium-binding proteins [11]. Three calcium-binding polypeptides were observed in the photosystem II preparations (fig.1B). Two polypeptides of photosystem II membranes with apparent molecular masses of 26 and 24 kDa bind $^{45}Ca$ strongly (fig.1B). A third polypeptide with an apparent molecular mass of 33 kDa bound $^{45}Ca$ only very weakly. $^{45}Ca$ binding by the photosystem II polypeptides is clearly highly selective for $Ca^{2+}$ since the assay was performed in the presence of 5 mM $Mg^{2+}$ and 60 mM $K^+$; lowering $Mg^{2+}$ levels to 0 mM had no apparent impact on the level of $^{45}Ca$ binding by the immobilised proteins (not shown).

The photosystem II calcium-binding polypeptides of 26 and 24 kDa have the same mobility on SDS-PAGE as polypeptide components of the LHCII complex. To determine if the calcium-

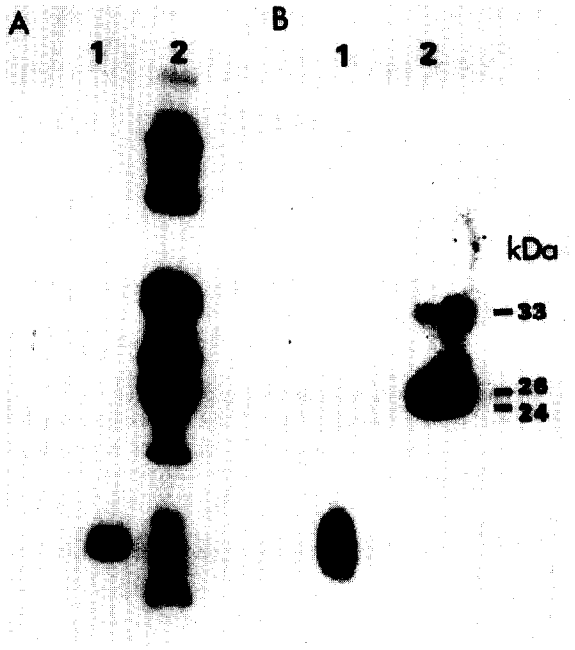


Fig.1. Identification of calcium-binding polypeptides of photosystem II on nitrocellulose membrane after separation by SDS-polyacrylamide gel electrophoresis. (A) Coomassie-stained gel of calmodulin (lane 1) and photosystem II polypeptides (lane 2). (B) Autoradiograph of the polypeptides of A immobilised to nitrocellulose after incubation with $^{45}Ca$ and washing with 50% ethanol. Lanes: 1, calmodulin (2 $\mu$g); 2, photosystem II polypeptides (40 $\mu$g chlorophyll). Molecular masses of the calcium-binding polypeptides indicated were determined by comparison to the migration of polypeptides of known molecular masses.

binding polypeptides are components of the LHCII complex, photosystem II membranes were further solubilised using the detergent OGP [14] to separate the peripheral LHCII polypeptides and the core components of photosystem II. The isolated LHCII polypeptides and the polypeptides of the LHCII depleted photosystem II-core were resolved by SDS-PAGE, transferred to nitrocellulose membrane and incubated with $^{45}Ca$. Fig.2 shows that both the 24 and 26 kDa calcium-binding polypeptides co-isolate with the LHCII fraction, and are resolved by the presence of 5 M urea in the resolving gel. This agrees with experiments of Davis and Gross [19] that an isolated LHCII complex can bind calcium very specifically and further shows that calcium binding is restricted to only two discrete polypeptides of the isolated complex.

Photosystem II polypeptides resolved by SDS-polyacrylamide gel electrophoresis were transferred to PVDF membrane and the 24 and 26 kDa polypeptides subjected to N-terminal amino acid sequencing. The 27 kDa polypeptide was found to be blocked at the amino-terminus. The sequence of the first 23 amino acids of the 24 kDa polypeptide is presented in fig.3. The amino acid sequence was found to be highly homologous to the amino acid sequence derived from type II *cab* genes of petunia [20], tomato [21] and lemna [22] (fig.3). This is the only reported N-terminal amino acid sequence for the product of a type II *cab* gene and indicates that the stromal processing site of the type II *cab* gene product is located between amino acid residues 44 and 45 of the pre-protein. This would make the mature type II *cab* gene product 7 amino acids smaller than previously predicted [22]. This may partly explain the increased mobility of this polypeptide during SDS-PAGE relative to the major products of the type I *cab* genes.

The location of any calcium-binding sites in the 26 and 24 kDa polypeptides is uncertain since no homology can be found between the derived amino acid sequence of these polypeptides and sequences of known calcium-binding proteins. Phosphorylation of LHCII in the light may provide the polypeptides with phosphate ligands to calcium. However, the 24 kDa polypeptide is not phosphorylated [23] and we observed no effect on calcium-binding following incubation of thylakoid membranes with ATP, prior to isolating the photosystem II complex. It is most probable that carboxyl side-groups within the polypeptides are involved in calcium binding. We have incubated photosystem II membranes with the hydrophobic carbo-diimide, DCCD, that reacts with carboxyl side-groups within hydrophobic regions of proteins. Fig.2B (lane 4) shows that DCCD almost completely inhibits calcium binding to both the 24 and 26 kDa polypeptides. This indicates that the calcium-binding ligands are carboxyl groups within normally hydrophobic regions of the proteins. Labelling of the proteins with $^{14}$C-DCCD and subsequent sequencing might allow the calcium-binding region to be identified.

A third calcium-binding polypeptide of 33 kDa was also detected in some experiments, although it bound $^{45}$Ca only very weakly and was not observed when urea was present during SDS-PAGE. A

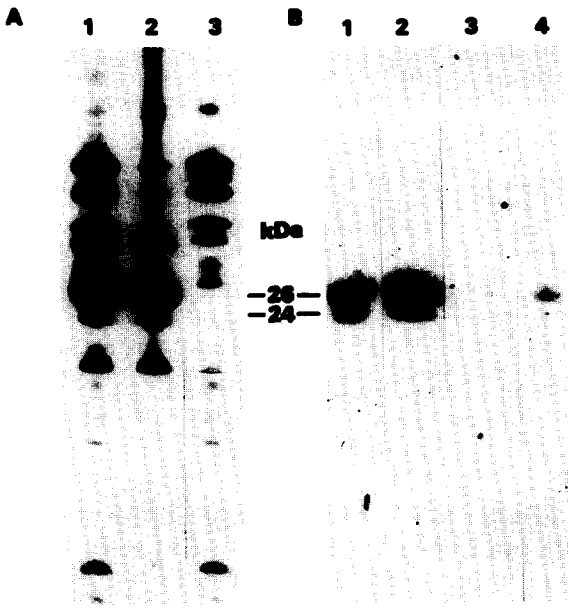


Fig.2. Identification of calcium-binding polypeptides present in the LHCII and photosystem II-core complexes prepared by solubilisation of photosystem II preparations with OGP. (A) Lanes: 1, photosystem II; 2, LHCII; 3, photosystem II core. (B) Autoradiogram of polypeptides of A immobilised onto nitrocellulose and incubated with $^{45}$Ca. Lane 4, autoradiograph of DCCD-treated membranes of A, lane 1.

number of polypeptides of photosystem II migrate with apparent molecular masses between 32 and 34 kDa. Because of the weak binding of $^{45}$Ca it was not possible to determine if this polypeptide was a component of the oxygen-evolving complex by Tris washings. It is interesting to note, however, that calcium-binding sites on the 33 kDa polypeptide of the oxygen-evolving complex and the 32 kDa reaction center polypeptide have been predicted by comparison of their derived amino acid sequences to that of a known mammalian calcium-binding protein [24]. The amino acid sequence of the 33 kDa polypeptide of the oxygen-evolving complex, derived from the pea cDNA sequence [25], shows a sequence of 27 amino acids, also conserved in other plant species, with all the structural requirements required for the formation of a calcium-binding EF hand typically found in calcium-binding proteins such as calmodulin and intestinal calcium-binding proteins. Although the 32 kDa D1 polypeptide sequence shows some homology to calcium-binding proteins it would not be predicted to form an EF hand tertiary structure

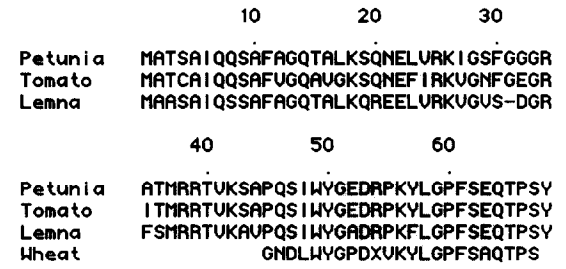


Fig.3. Comparison of the N-terminal 68 amino acids derived from the type II *cab* genes of petunia [20], tomato [21] and lemna [22] with the N-terminal amino acid sequence obtained from the 24 kDa wheat polypeptide. A dash indicates a gap introduced into the sequence to provide maximal alignment of the sequences. An X indicates a residue that could not be positively identified.

and is not such a strong candidate for the (33 kDa) calcium-binding protein identified here. However, high affinity calcium binding to an intrinsic photosystem II polypeptide, such as D1 or D2, may require stabilisation through interaction with hydrophilic polypeptides of the oxygen-evolving complex; the technique used here would not be applicable for the detection of such calcium binding. Further work on calcium binding to the 33 kDa polypeptide of the oxygen-evolving complex is required to show conclusively that it is a calcium-binding protein.

This report presents the direct observation of calcium binding by individual polypeptides of photosystem II. We have shown that two polypeptides of 24 and 26 kDa bind calcium strongly and are probably individual polypeptides of the LHCII complex. The functional role of calcium binding by the 24 and 26 kDa polypeptides is unknown. An unidentified polypeptide of 24 kDa has been shown to be located in close proximity to the oxygen-evolving complex [26] and may be the LHCII calcium-binding polypeptide.

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