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Removal of nuclear contaminants and of non-specifically photosystem II-bound copper from photosystem II preparations

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In conventional photosystem II preparation high amounts of Cu are found. After fractionation by centrifugation, Cu can be completely removed from photosystem II without affecting either its photosynthetic activity or the composition of its specific proteins. We could demonstrate that the Cu was associated with nuclear contaminants in the starch fraction. Among the contaminants, several histones were identified by specific antisera and by N-terminal sequencing. In order to obtain homogeneous BBY preparations of PSII a procedure is employed that involves a 10000 ^ centrifugation step and which eliminates non-specifically bound metals, nucleic acids and histones with the starch pellet. The resulting starch-free BBY (BBY,_), which is free of these nuclear contaminants, is an appropriate preparation for biophysical studies or for those of metal interactions with PSII.

Key words - Copper, histones, pea, photosystem II, Pisum sativum, spinach, Spinacia oleracea.

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Introduction

One of the events that have led to a better understanding of the primary steps involved in photosynthetic electron transport has been the development of isolation methods for the individual multienzyme complexes involved in such a process: photosystem II (PSII), the cytochrome b_6/f complex and photosystem I (PSI).

Regarding PSII, different isolation procedures have been developed, based mostly on detergent solubilization (Berthold et al. 1981, Dunahay et al. 1984) but also on mechanical fractionation (Svensson and Albertsson 1989) of the thylakoid membrane. Nevertheless, to en-able comparison of the results reported by different groups, it is important to obtain standardized, highly purified preparations with the same protein constituents and mineral cot'actors such as manganese, copper and calcium. This is particularly important for sophisticated biophysical studies performed on PSII. The method de-scribed by Berthold et al (1981), the so-called BBY preparation, using Triton X-100, has become standard and is used extensively in biochemical and biophysical studies of PSII. However, regarding the Cu content (Droppa and Horvath 1990) and the polypeptide pattern of PSII in the 10 to 20 kDa region (Baron ct al. 1990, Arellano et al. 1992), large variations have been reported. In a previous study we found that by differential centrifugation virtually all Cu could be removed (Baron et al. 1993). In this work we identity Cu in the photosystem II preparation as being associated with nuclear contaminants. Histones, nucleic acids and other metal ions are precipitated with starch upon centrifugation. Removal of these contaminants by introducing a starch centrifugation at 10000 g, yielded highly active PSII, starch-free BBY particles (BBY_{s-}) devoid of Cu, and with improved optical properties. It is therefore suggested that Cu is not directly involved in oxygen evolution or in electron flow through PSII, and the BBY_{s-} preparation is thus the most appropiate for studies of metal interactions with PSII.

Abbreviations - BBY, PSII preparations isolated as described by Berthold et al. (1981); BBY_{s+}, starch-containing BBY; BBY_{s-}, starch-free BBY; CP 14, Chl a/b binding polypeptide in PSII described by Irrgang et al. (1990); ELIPs, early light-inducible proteins; OEC, oxygen evolving complex; SF-proteins, polypeptides associated with the starch pellet in the BBY isolation process.

Materials and methods

Pea plants (*Pisum sativum* L. cv. Lincoln) were grown hydroponically in Hewitt's full nutrient solution in a growth chamber for 4 weeks as described in Baron and Sandmann (1988). Spinach (Spinacia oleracea L.) was obtained from the local market.

BBY particles were prepared essentially according to Berthold et al. (1981) with the modifications of Ford and Evans (1983). BBY_{s-} were obtained by adding a new step in the isolation method: after incubation of purified thylakoids with Triton X-100, the solution was centrifuged for 4 min at 10000 g to obtain a greenish-white starch pellet. The supernatant without starch was centrifuged at 40000 g for 25 min and the PSII particle-containing pellet resuspended in 50 mM, MES, pH 6.5, 5 mM $MgCI_2$, 15 mM NaCI and 400 mM sucrose (stock buffer). To obtain BBY_{s+} preparations, the centrifugation step at 10000 g after Triton X-100 treatment was omitted. In both cases, excess detergent was removed from the PSII particles by washing twice with the stock buffer. For the study of contaminants, the starch pellet was washed 3x with stock buffer, centrifuging for 5 min at 5000 g each time. Particles devoid of polypeptides from the oxygen evolving complex (OEC) were obtained by washing as described in Ljungberg et al. (1986).

Sodium dodecyl sulphate-polyacrylamide gel electro-phoresis (SDS-PAGE) was carried out on 17.5% polyacrylamide gels containing 4 M urea, as described by Laemmli (1970).

For raising antisera against the proteins present in the starch fraction, the proteins were isolated by cutting the stained protein bands from SDS-polyacrylamide slab gels, and eluting the slices by electrodialysis. About 10 µg of each protein was used for immunizing mice, using standard immunological procedures (Fraser et al. 1992). Western blots were performed as in Towbin et al. (1984), and the antigen-antibody complex was developed by a peroxidase-conjugated goat-antimouse serum. Antibodies against the starch fraction proteins and ELIPs (early-light-inducible proteins; Cronshagen and Herzfeld 1990) were obtained for pea. The antisera against OEC proteins, CP 14 (Irrgang et al. 1990), phosphoprotein and the 10 kDa protein described by Ljungberg et al. (1986), were raised from spinach proteins. Histone antisera were against animal histones.

For N-terminal sequencing, proteins were isolated by SDS-PAGE under the above conditions without urea, and transferred to a polyvinylidene difluoride membrane (PVDF, Millipore) using a JKA-Biotech (Bedford, USA) semidry electroblotter. After Coomassie staining of the PVDF membrane, protein bands were excised and se-quenced on an Applied Biosystem (La Jolla, CA, USA) model 470 Sequenator.

The isolation of DNA fragments by electrophoresis in 0.3% agarose gels, has been described by Sambrook et al. (1989). Nucleic acid concentration was spectrophotomet-rically determined at 260 nm. The absorbance ratio A261/A280 was also measured to identify protein contam-ination.

The metal content was determined by atomic absorp-tion spectrometry (Perkin-Elmer model 503), using a hollow cathode lamp and a graphite furnace (Perkin-Elmer HGA-400; Uberlingen, Germany) (Arellano et al. 1993).

Results

Polypeptide patterns of thylakoid membranes, BBY_{s+} and BBY_{s-} and the starch pellet of the BBY_{s-} isolation, both from spinach (lanes 1-4) and pea (lanes 7-10), are shown in Fig. 1. Pea and spinach extrinsic 16, 23 and 33 kDa proteins related to the OEC were applied as reference proteins in lanes 5 and 6. They were obtained from the supernatant of Nad-treated PSII particles. Higher migra-tion rates of the extrinsic 16 and 23 kDa proteins for pea, as compared to spinach, were noted. This means a lower apparent molecular mass of the former. It is seen that pea BBY,,, (lane 8) is depleted of 4 polypeptides of apparent molecular masses of ca 12, 17, 19 and 20 kDa. The missing polypeptides appear in the starch traction pellet resulting from the 10000 g centrifugation step (lane 7). Parallel results were. obtained with spinach, with starch fraction proteins (SF-proteins) within the same molecular mass range. The identity of SF-proteins of both species was demonstrated by means of cross reactivity with the corresponding antisera (data not shown). The weakly-stained

polypeptides which appear between 12 and 17 kDa in the pea starch fraction, are easily removed by washing of the starch pellet as described in Materials and methods. It appears that in spinach BBY,,, preparations, only 3 polypeptides are lost by starch removal. However, we found by immunodetection that the 20 kDa SF-protein in spinach BBY..+ comigrates with the OEC 16 kDa pro-tein (Fig. 2). We carried out western blotting with differ-ent spinach preparations: isolated thylakoids (lanes 1 and 10), BBY,+ (lanes 2, 9), BBY,. (lanes 3, 8), starch pellet fraction (lanes 4, 7) and the supernatant from NaSCN/ Triton X-100 treated BBY.,- (lanes 5, 6). After transfer to/^ nitrocellulose, lanes 1 to 5 were stained with Ponceau red and lanes 6 to 10 were treated with a specific antiserum against the OEC 16 kDa protein. A positive immunoreactive band was detected in lanes 10, 9, 8 and 6, corresponding to spinach thylakoids, BBYs+, BBY,_ and the supernatant from Triton NaSCN-treated BBY,-, respec lively. The latter treatment solubilizes the OEC polypep-tides, including the 10 and 22 kDa proteins (Ljungberg et al. 1986). It is pointed out that cross reactivity with the SF-proteins was not observed.

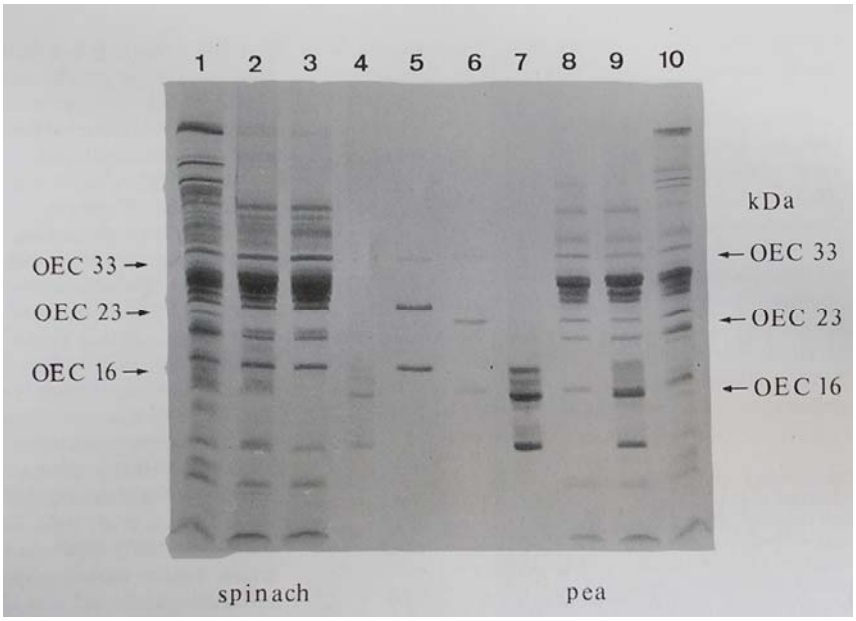

Fig1 SDS-4 M urea PAGE of Thylakoids (1,10), BBY_{s+} (2,9), BBY_{s-} (3,8), starch pellet (4,7) and the supernatant from NaCl- treated BBY_{s-} (5,6)

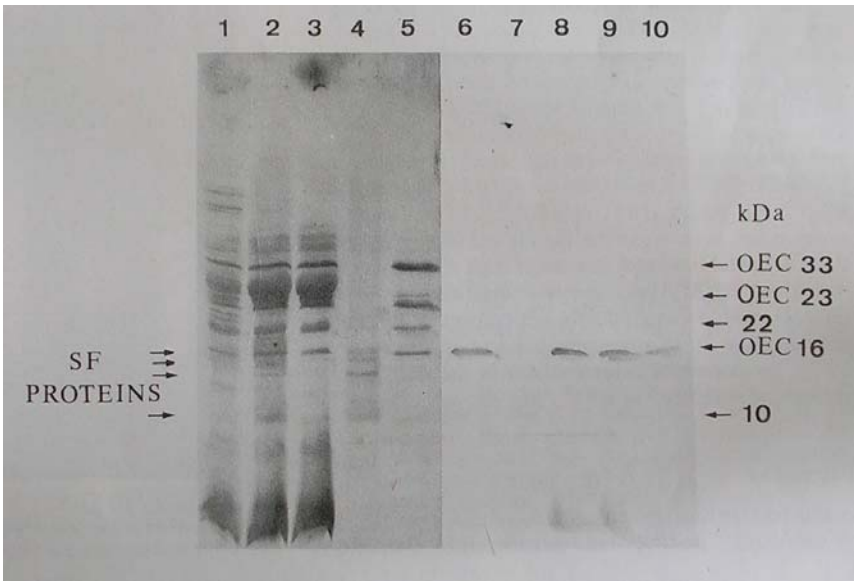

Fig. 2. Reactivity of the antibody against OEC 16 kDa protein with blotted samples (lanes 6-10), blotted proteins bands stained by Ponceau red (lanes 1-5).
Spinach samples of thylakoids (1, 10), BBY_{s+} (2,9), BBY_{s-} (3,8), starch fraction (4,7) and the supernatant from NaSCN/Triton-Treated BBY_{s-} (5,6)

To characterize the 4 proteins that appear in the starch traction, we raised different antibodies against PSII proteins with molecular masses in the 10-20 kDa range. Antisera against CP 14 apoprotein, 10 kDa phosphoprotein, the 10 kDa protein described by Ljungberg et al. (1986) and the ELIPs, did not show cross-reactivity with the starch-associated proteins from pea or spinach by immunoelectrophoresis. In addition, we carried out west-ern blotting experiments with antisera raised against our SF-proteins. The BBY_{s-} preparation, tentatively free from these proteins, and with good PSII-related activity, gave no immunoreactive bands, indicating that our polypep-tides were neither PSII proteins nor their degradation products. For the identification of the starch-associated proteins, we tried to determine their N-terminal sequence. Unfortunately, 3 of the proteins were N-terminally blocked. Thus, it was possible to obtain only the sequence of the 17 kDa protein: Ala-Arg-Tre-Lys-Gln. Comparing with homologous proteins in the GenBank Swiss-Prot, we found that our protein was identical to histone H3 from plants, fungi, yeast and humans.
The results prompted us to test the identity of the other SF-proteins with histone antibodies. Western blot analysis with antibodies against animal histones confirmed the identity of the sequenced protein and identified SF 20 and SF 19 as H2 histones, and SF 12 as histone H4. Figure 3 shows that in the starch fraction from spinach, the antiserum against H2b cross-reacted with SF 20 and SF 19, whereas the antiserum against H4 reacted with SF 12 and had a secondary reaction with the SF 17 kDa polypeptide.

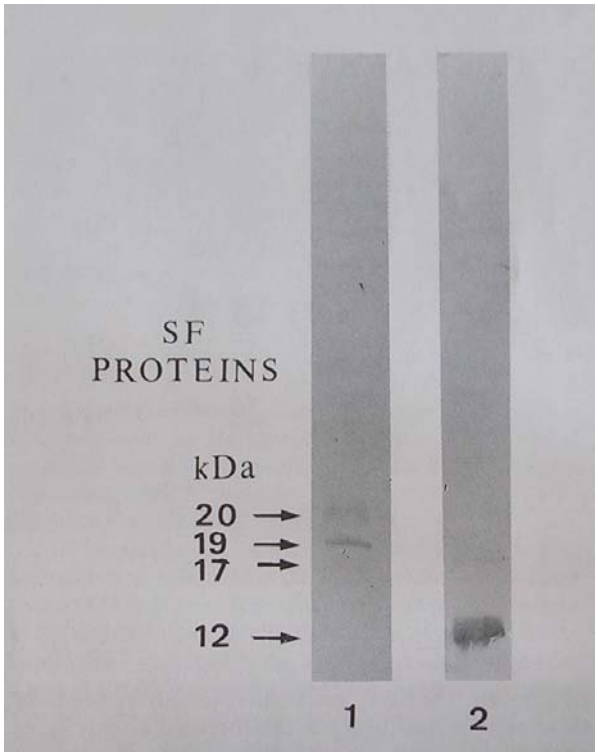

Fig. 3. Inmunoblotting of a pea starch fraction using antibodies against animal histones H2b (1) and H4 (2).

The identification of the histones in the usual BBY preparation (BBY_{s+}) raised the question as to whether or not they were associated with DNA. We therefore deter-mined the nucleic acid content of spinach BBY_{s+} and BBY_{s-} particles, and found it to be 150 and 20 ($\mu g\ mg^{-1}$ Chl, respectively. A large amount of nucleic acids (DNA and RNA) was found in BBY_{s+}, which was decreased 7.5-fold in BBY_{s-}. The separation of DNA fragments and RNA from spinach BBY_{s+} and BBY_{s-}, as well as

in the starch pellet, is shown in the agarose gel of Fig. 4. BBY_{s+} (lane 1) contains a large amount af high-size DNA fragments and RNA. These decrease drastically in BBY_{s-} (lane 2), with a concomitant high content in the starch fraction (lane 3). In a previous publication (Baron et al.

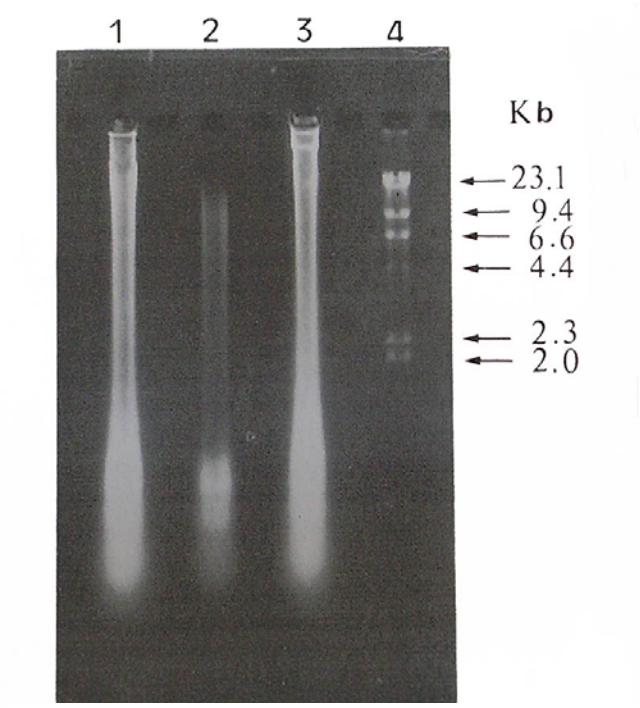

Fig. 4. Agarose gel analysis of spinach BBY_{s+} (1), BBY_{s-} (2), starch fraction (3) and Phage λ digested with Hind III as marker (4)

1993), the decreased content of Cu in the BBY_{s-} preparations as compared to BBY_{s+} was shown.

Discussion

A standard method for preparation of PSII particles, the so-called BBY particles, was introduced by Berthold et al. (1981), resulting in a PSII complex being obtained of high yield with a high oxygen-evolving ability. In the course of our work on the binding of Cu to this prep-aration, we observed that after a 10000 g centrifugation to remove starch the pellet also contained high amounts of DNA, RNA, Cu and 4 polypeptides of ca 12, 17, 19 and 20 kDa (SF-proteins). The resulting PSI1 particles, free from these contaminants, were called BBY_{s-}. By using immunological methods and after protein sequencing of one of these proteins, it could be demonstratet that SF-12, 17, 19 and 20 kDa were not PSI1 proteins, but were histones closely related to histones H2, H3 and H4 (Spiker 1982, Spiker 1985). Centrifugation at lower than 10000 g did not completely remove these contamination proteins. There is a progressive decrease of Cu content and SF-proteins following centrifugation at 1000 g , 3000 g or 5000 g, (Baron et al. 1993). The different procedures for obtaining BBY particles (Ford and Evans 1983, Droppa et al. 1984, Dunahay et al. 1984, Sibbald and Green 1987) either do not all include the centrifu-gation step for removing starch or are performed at in-sufficient speed.

Thylakoid membranes obtained by differential centrifugation are usually contaminated by nuclear fragments (Hoyer-Hansen and Simpson 1977, Welty et al. 1992). Such preparations are commonly used for BBY isolation without previous purification. Our present work shows that nuclear

contaminations are found in the conventional BBY preparations used in most studies of PSII. Morever, the histones found in these preparations produce a false PSII polypeptide pattern, and in some cases the histones even comigrate with functional PSII proteins (e.g. SF 20 with OEC 16, Figs 1 and 2). Some authors have isolated BBY particles using low speed centrifugation for starch removal (1000 or 3500 g), but our results demonstrate that BBY_{s-} can only be obtained after centrifugation at 10000 g which removes not only starch, but also histones and DNA. If, according to Welty et al. (1992), the his-tones are maintained in a chromatin structure during preparation of the thylakoids, they would pellet after the Triton X-100 treatment at the centrifugal force in question.

As regards the high metal content of the starch-histone contaminated BBY_{s+} particles, the bulk of Cu is removed in the BBY_{s-}, with its concomitant appearance in the starch fraction, where both histones and nucleic acids are present. We have found high amounts of Ca, Fe and Zn in the starch pellet (data not shown), which may contam-inate the BBY_{s+} preparation.

Among the authors who have described the presence of metals in cell nuclei bound to nuclear proteins and/or nucleic acids are Lebkowski and Laemli (1982a,b). Fe, Cu, Mg and Zn have been detected in situ in the nucleus of animal cells by X-ray microanalysis or by X-ray fluo-rescence analysis of a native chromosomal DNA-protein complex (Skrinska et al. 1978, Quintana et al. 1987). The phosphate groups as well as the purine and pyrimidine bases of the nucleic acids offer a large number of binding sites for metal ions (Eichhorn et al. 1971). Moreover, metals like Cu and Ca could be involved in the reversible process of DNA folding (Lebkowski and Laemli 1982a,b).

Based on the present results, we propose that the Cu found in the BBY_{s+} preparation is a nuclear contamination. It can easily be removed from the PSII complex by removal of starch, a treatment that exerts no negative effect on the oxygen-evolving activity (Baron et al. 1983) and thus suggesting that this metal is not directly in-volved in oxygen evolution. Consequently, several reports (Droppa et al. 1984, Ono et al. 1984, Sibbald and Green 1987, Droppa and Horvath 1990, Arvidsson et al. 1992) on the association of Cu with PSII need to be reevaluated. Manganese, a metal directly involved in ox-ygen evolution, remains bound to the PSII complex. Binding of other metal ions to PSII may also have to be re-examined as a result of possible contamination by DNA and histones. The use of a preparation similar to our BBY_{s-} may be necessary to obtain reliable results concerning the interaction of metals with PSII.

Our notion that the Cu found in BBY_{s+} cannot have another origin, for example Cu-binding proteins in the chloroplast or cytosol, is based on previous observations (Baron et al. 1993). We demonstrated that these proteins were removed during the first steps of the PSII isolation process, with a parallel decrease in Cu.

The introduction of the 10000 g centrifugation step with the concomitant removal of starch, nucleic acids and histones, also changes the optical properties of the BBY particles: transmittance at 830 nm is increased (data not shown) as a consequence of lesser aggregation of the samples.

The method for removing nuclear contamination from PSII preparations also has the advantage of not reducing the preparative yield and not prolonging isolation. Other means of eliminating impurities, such as the use of chloroplasts purified by a discontinuous sucrose gradient, involve excessive time and effort.

In conclusion, the simple modification proposed for the BBY procedure for the isolation of PSII particles, which involves an additional centrifugation for 4 min at 10000 g after Triton treatment, increases the purity of PSII. The resulting BBY_{s-} particles are a highly purified PSII preparation, which is of advantage for metal binding studies and for biophysical studies on PSII.

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