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Isolation of lumenal proteins from spinach thylakoid membranes by Triton X-114 phase partitioning

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Abstract

The proteins present in the thylakoid lumen of higher plant chloroplasts have not been rigorously examined. In this communication we present a simple and rapid procedure for the isolation of the soluble proteins and extrinsic membrane proteins present in the thylakoid lumen from spinach. Our procedure involves extensive washing of the thylakoid membranes followed by Triton X-114 phase partitioning. When analyzed by one-dimensional polyacrylamide gel electrophoresis (PAGE), we obtain results which are very similar to those obtained by Kieselbach et al. using more classical methods [T. Kieselbach, A. Hagman, B. Andersson, W.P. Schroder, J. Biol. Chem. 273 (1998) 6710–6716]. About 25 major proteins are observed upon Coomassie blue staining. Upon two-dimensional isoelectric focusing–sodium dodecyl sulfate–PAGE and either Coomassie blue or silver staining, however, numerous other protein components are resolved. Our findings indicate that the total number of proteins (soluble and extrinsic membrane) present in the lumen may exceed 150.

Keywords: Lumenal protein; Chloroplast; Triton X-114 phase partitioning

1. Introduction

Biological membranes are involved in virtually every aspect of cellular organization and activity. One of the most intriguing aspects of membrane function is its role in the mediation of energy transduction in photosynthetic organisms. While much progress has been made in the understanding of photosynthetic processes, the mechanisms involved in protein processing, membrane insertion, cofactor assembly and regulation of photosynthetic electron transport remain poorly understood.

In this communication, we have developed a method to isolate both the soluble proteins and extrinsic membrane proteins present in the thylakoid lumen from higher plants. This subcellular compartment has not been rigorously examined. We hypothesize that components located in the lumen may be involved in the assembly of the photosynthetic apparatus. Our results indicate that there may be more than 150 protein components present in the lumenal compartment; the functions of less than 15 of these

Abbreviations: IEF, isoelectric focusing; LiDS, lithium dodecyl sulfate; MSP, manganese stabilizing protein; PAGE, polyacrylamide gel electrophoresis; RuBp carboxylase, ribulose bisphosphate carboxylase; SDS, sodium dodecyl sulfate; TX-114, Triton X-114

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have been identified. Some of the components which have been identified include extrinsic structural proteins involved in photosystem II (PS II) (manganese stabilizing protein (MSP), 24 and 17 kDa proteins; reviewed in [1]), electron transport proteins that shuttle electrons between the cytochrome b_6/f complex and photosystem I (PS I) (plastocyanin and cytochrome c_{553}), processing enzymes involved in the post-translational modification of PS II proteins (such as ctpA [2], and components which may be involved in protein folding [3]).

Recent studies have isolated lumenal protein fractions by extensive washing of thylakoid membranes followed by sonication [14] or Yeda press [4] treatments followed by ultracentrifugation. Rather than utilize mechanical disruption to release the lumenal components, our procedure utilizes Triton X-114 (TX-114) phase partitioning. It is quick and relatively easy to perform, does not require a specialized apparatus (such as a Yeda or French press), does not require ultracentrifugation steps and yields both soluble lumenal proteins and lumenally exposed extrinsic membrane protein components. Additionally, this method may be suitable for the isolation of these components from relatively small amounts of tissue and could be applied to the isolation of this protein fraction from *Arabidopsis* or *Chlamydomonas* chloroplasts.

2. Materials and methods

Class II chloroplasts, which lack the outer chloroplast envelope but which maintain thylakoid integrity, were isolated from washed and deveined spinach leaves by blending with ice-cold 100 mM sucrose, 200 mM NaCl, 5 mM $MgCl_2$, 50 mM Na-K phosphate, pH 7.4 for three, 3 s bursts. The blended leaves were then filtered through two layers of cheese cloth over Miracloth (Calbiochem. Corp.). The chloroplasts were harvested by centrifugation at $2400\times g$ for 5 min. The chloroplast pellet was suspended to a chlorophyll concentration of 200 μg/ml in 0.33 M HEPES–NaOH, pH 8.0, 10 mM $CaCl_2$ with glass homogenization. After centrifugation at $7500\times g$ for 5 min, the chloroplast pellet was suspended to a chlorophyll concentration of 200 μg/ml in 10 mM sodium pyrophosphate, pH 7.8 with glass homogenization. After centrifugation at $7500\times g$ for 5 min, the chloroplast pellet was suspended to a chlorophyll concentration of 200 μg/ml in 2 mM Tricine, pH 7.8, 300 mM sucrose with glass homogenization. After centrifugation at $7500\times g$ for 5 min, the chloroplast pellet was suspended to a chlorophyll concentration of 200 μg/ml in 30 mM Na-K phosphate, pH 7.8, 50 mM NaCl, 5 mM $MgCl_2$, and 100 mM sucrose with glass homogenization. These washes were then repeated. After the final wash the washed thylakoids were resuspended in 0.33 M HEPES–NaOH, pH 8.0, 10 mM $CaCl_2$ to a chlorophyll concentration of 0.2–1.0 mg/ml. All procedures up to this point were performed at 0–4°C. These washes are essentially as described by Kieselbach et al. [4].

To test for intactness, washed thylakoid membranes were treated with thermolysin. The washed membranes were suspended in 0.33 M HEPES–NaOH, pH 8.0, 10 mM $CaCl_2$ at a chlorophyll concentration of 0.2 mg/ml. Samples were incubated for 2 h at 24°C with either 100 μg/ml thermolysin or 100 μg/ml thermolysin+1% TX-114. At the end of the incubation, the samples were placed on ice and brought to 20 mM EDTA. TX-114 was added to the 100 μg/ml thermolysin-only sample to a final concentration of 1%. Phase partitioning was then performed as described below.

TX-114 phase partitioning was performed essentially according to Bricker and Sherman [5]. The washed thylakoid membranes were brought to 1% TX-114. The solubilized membranes were then incubated on ice for 5 min and then incubated at 37°C for 5 min. During this incubation the clear detergent solution became very cloudy due to the condensation of TX-114 micelles. After centrifugation at $14\,000\times g$ for 5 min at room temperature, the solution exhibited phase separation with a dark green, detergent-enriched lower phase and a pale green, detergent-depleted upper phase. The upper phase was carefully collected, brought to 1% TX-114 and the phase partitioning process repeated. After the second phase partitioning, the upper phase was colorless and contained the lumenal proteins. A flow chart summarizing the steps in this lumenal protein isolation procedure is shown in Fig. 1.

Two-dimensional (2D) electrophoresis was performed by the method of O'Farrell [6]. Isoelectric focusing (IEF) was performed in 2 mm glass tubes

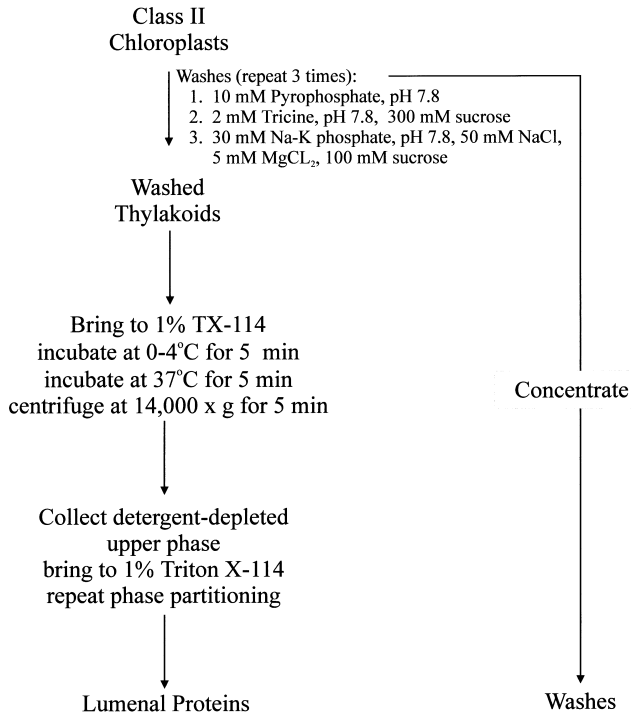

Fig. 1. Flow chart for the isolation of thylakoid lumenal proteins. The lumenal fraction contains both soluble lumenal proteins and lumenally exposed extrinsic membrane components. Samples from the first three washes were pooled and concentrated to yield the washes fraction. PAGE analysis of the various fractions is shown in Figs. 3 and 4.

using 2% pH 4–8 ampholines for 9600 V h. An IEF internal marker, tropomyosin, was added to the sample. This protein migrates as a doublet, with the lower polypeptide spot migrating at 33 kDa with a p*I* of 5.2. After IEF, the gel was equilibrated in 10% glycerol, 50 mM dithiothreitol (DTT), 2.3% sodium dodecyl sulfate (SDS) and 0.0625 M Tris–HCl, pH 6.8. After equilibration, the IEF gel was sealed with agarose to the top of a stacking gel which overlaid a 12.5% resolving gel. SDS–polyacrylamide gel electrophoresis (PAGE) was carried out for 4 h at 12.5 mA/gel. Molecular weight standards were included in the agarose and appear as horizontal lines in the 2D gel. The 2D gel was either stained with Coomassie blue or with silver [7].

One-dimensional (1D) lithium dodecyl sulfate (LiDS)–PAGE was performed as previously described [8]. The chlorophyll concentration was determined by the method of Arnon [9]. Protein assays were performed by the bis-cinchoninic acid method [10]. Protein fractions were concentrated by centrifugal ultrafiltration. For gel documentation, wet or dry gels were scanned using a Microtek Scanmaker E6 digital scanner and the image acquired with PhotoImpact, Ver. 3.0 software (Ulead Systems). The identification of ribulose bis-phosphate carboxylase (RuBp carboxylase; large and small subunits), the MSP, and the 24 and 17 kDa proteins of photosystem II were made by comparison to the individual purified protein components.

3. Results and discussion

Intact thylakoid membranes are required for the isolation of lumenal protein components. In order to demonstrate the intactness of the thylakoid membranes isolated by this procedure, the washed thylakoid membranes were treated with thermolysin either in the absence or the presence of the detergent TX-114. After incubation with the protease, EDTA was then added to inhibit its activity and TX-114 was added to the sample which did not originally contain detergent. Both samples were then subjected to phase partitioning to isolate the lumenal components as described below. The presence of detergent during protease treatment was expected to render the lumenal components significantly more susceptible to the action of the protease. This was found to be the case. Fig. 2 demonstrates that a number of lumenal components are proteolytically degraded in the presence of TX-114 but not in its absence. This result indicates that the thylakoid membranes isolated by the washing procedure appear to be intact and that the lumenal components were protected from the action of the thermolysin. These results confirm the findings previously obtained for the washing procedures used in these studies [4].

Various protein fractions recovered during the lumenal isolation procedure outlined in Fig. 1 are shown resolved by 1D LiDS–PAGE in Fig. 3. The sequential washing of the thylakoids was highly effective in removing stromal components, stromally exposed extrinsic membrane proteins and cytoplasmic contaminants (Fig. 3, lanes A and B). RuBp carboxylase and numerous other proteins were efficiently removed from the thylakoid membranes by these washes (Fig. 3, lane C). These results are essen-

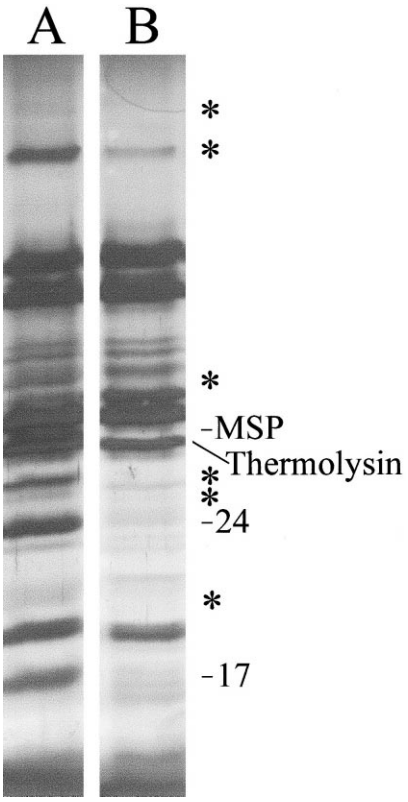

Fig. 2. Thermolysin treatment of washed thylakoid membranes. (A) Lumenal fraction isolated from membranes incubated with 100 μg/ml thermolysin; (B) lumenal fraction isolated from membranes treated with 100 μg/ml thermolysin in the presence of 1% TX-114. The presence of TX-114 in (B) disrupts the thylakoid membrane and exposes the lumenal components to the action of the protease. Shown is a silver stained 15% polyacrylamide gel. Proteins degraded by the protease are indicated (*). Some identified components are labeled to the right.

tially identical to those presented by Kieselbach et al. [4] (their Fig. 2, lanes 1 and 3).

TX-114 phase partitioning has been shown to be an extremely efficient method for separating soluble and extrinsic membrane proteins from intrinsic membrane components [11] and was shown to be applicable to plant and cyanobacterial thylakoid membrane systems [5,8]. Membranes are first solubilized in a TX-114 solution at 0–4°C and then subjected to phase partitioning at 30–37°C. After centrifugation, a detergent-enriched phase containing integral proteins separates from a detergent-depleted phase containing soluble proteins and extrinsic membrane components (reviewed in [12]). We reasoned that treatment of washed thylakoid membranes with TX-114 followed by phase partitioning would release the soluble lumenal proteins and lumenally exposed extrinsic membrane proteins into the detergent-depleted phase.

Fig. 3 shows the results of such an experiment in which the proteins of the various fractions were resolved by LiDS–PAGE. Washing of native thylakoid membranes (lane A) removed RuBp carboxylase and a variety of other components (lane C), yielding washed thylakoid membranes (lane B). TX-114 phase partitioning of the washed membranes yielded a detergent-enriched fraction containing the integral membrane proteins (data not shown) and a detergent-depleted fraction containing lumenally localized soluble proteins and lumenally exposed extrinsic membrane proteins (lane D). These lanes (A–D)

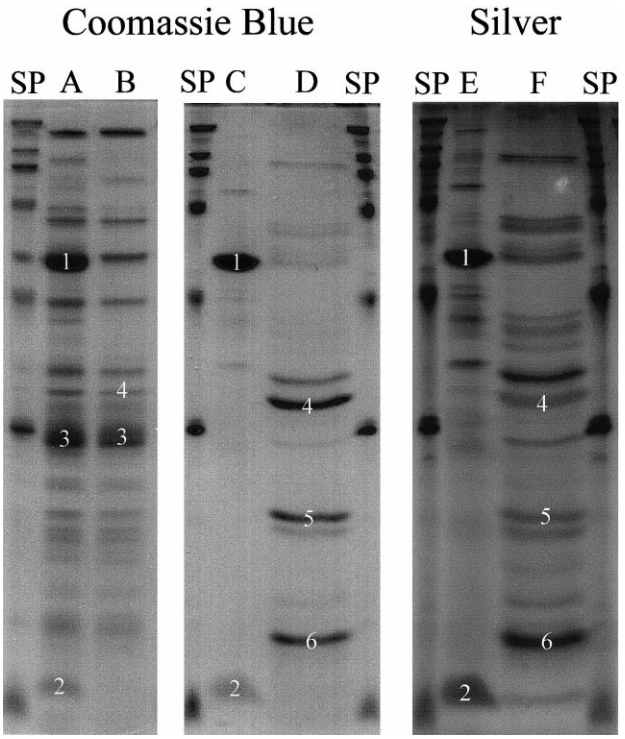

Fig. 3. Isolation of lumenal components by TX-114 phase partitioning. Lane A, unwashed thylakoid membranes; lane B, washed thylakoid membranes; lane C, pooled washes; lane D, lumenal fraction. Lanes A–D were stained with Coomassie blue. Lanes E and F are the pooled washes and the lumenal fraction, respectively, stained with silver. Some identified proteins are indicated as follows; 1, RuBp carboxylase, large subunit; 2, RuBp carboxylase, small subunit; 3, light-harvesting chlorophyll a/b protein; 4, photosystem II MSP; 5, photosystem II 24 kDa protein; 6, photosystem II 17 kDa protein. Shown is a 15% polyacrylamide gel. SP, standard proteins.

were stained with Coomassie blue. Lanes E and F show the washes and lumenal fractions stained with silver. On the original polyacrylamide gel, 25 protein bands were visible in the lumenal fraction when stained with Coomassie blue. These results are quite similar to those obtained by Kieselbach et al. [4]. Comparison of their data (their Fig. 2, lane 4) with those reported in this study (Fig. 3, lane D) indicates that very similar populations of lumenal proteins were isolated by these two procedures. Since both soluble and extrinsic membrane lumenal components are isolated by our procedure, quantitative differences are, however, evident. The major extrinsic proteins associated with PS II (MSP, 24 and 17 kDa polypeptides, for instance) are present in larger quantities than observed by Kieselbach et al. [4]. In their procedure, only the pool of these proteins unassociated with functional PS II centers (about 10% of the total amount of these components) was observed [13].

Our results, however, appear to indicate that there may exist a significantly larger population of lumenal components than previously observed. Silver staining of 1D separations of our preparation indicates that about 50 proteins can be resolved on the original gels. Kieselbach et al. [4] reported that they identified only five additional protein bands upon silver staining of 1D separations of their lumenal preparation. One possible explanation for this discrepancy is that our procedure isolated soluble proteins and extrinsic membrane proteins. The additional protein bands which we observe could be a population of extrinsic membrane protein components. We are currently investigating this possibility. It should be noted, however, that a number of protein components which have been assigned a lumenal location by other investigators [2,3] were not identified by Kieselbach et al. [4]. This may indicate that only the most abundant components were identified by these investigators and that a substantially larger population of low abundance proteins may be present in the lumen. Indeed, an even greater number of lumenal proteins can be readily resolved by IEF–SDS–PAGE (Fig. 4). Over 50 Coomassie blue stained spots are observed while over 150 silver stained spots are resolved. Please note that with either Coomassie blue or silver staining, the protein patterns obtained for the lumenal preparation (Fig. 4B,D) are highly complementary to the pattern obtained from the pooled washes of the thylakoid membranes (Fig. 4A,C). This would seem to indicate that the proteins observed are from two different populations and that we are not observing significant contamination of the lumenal fraction with proteins present in the washes. In our view, this is an important consideration. Recently, Peltier et al. [14] have presented the first proteomic study involving a survey of the proteins present in the thylakoid lumen. These authors also concluded that there was a large number of proteins present in the lumen. In this study, however, due to apparent incomplete washing of the thylakoid membranes, significant contamination of the lumenal protein fraction with stromal components occurred. For instance, one of the most abundant proteins present in their lumenal fraction appeared to be the large subunit of RuBp carboxylase. Other stromal components, such as aldolase and ferredoxin, were also prevalent. The presence of significant quantities of known stromal components in the lumenal fraction obtained by these authors, in our opinion, makes identification of actual lumenal proteins problematic.

It should be noted that there exists the formal possibility that extrinsic membrane protein components which are exposed to the *stroma*, but which are not removed by the extensive washing treatments which we have employed, would be recovered in the detergent-depleted lumenal fraction. This is a possible source of contamination in our procedure. The methods which utilize the mechanical disruption of the thylakoid membrane to release the lumenal components (sonication or Yeda press treatments) also suffer from similar sources of contamination. Kieselbach et al. [4], using Yeda press thylakoid disruption, reported significant contamination of their lumenal protein fraction with ferredoxin-$NADP^+$ reductase, a known stromally exposed membrane associated component. Peltier et al. [14], using sonication, recovered significant quantities of the α and β subunits of CF_1, which are known stromally exposed extrinsic membrane protein components.

In this communication we have presented a very rapid and simple procedure for the isolation of the soluble and extrinsic membrane proteins present in the spinach thylakoid lumen. Using these techniques, we have isolated the lumenal fraction from *Arabidopsis* and are currently identifying these protein com-

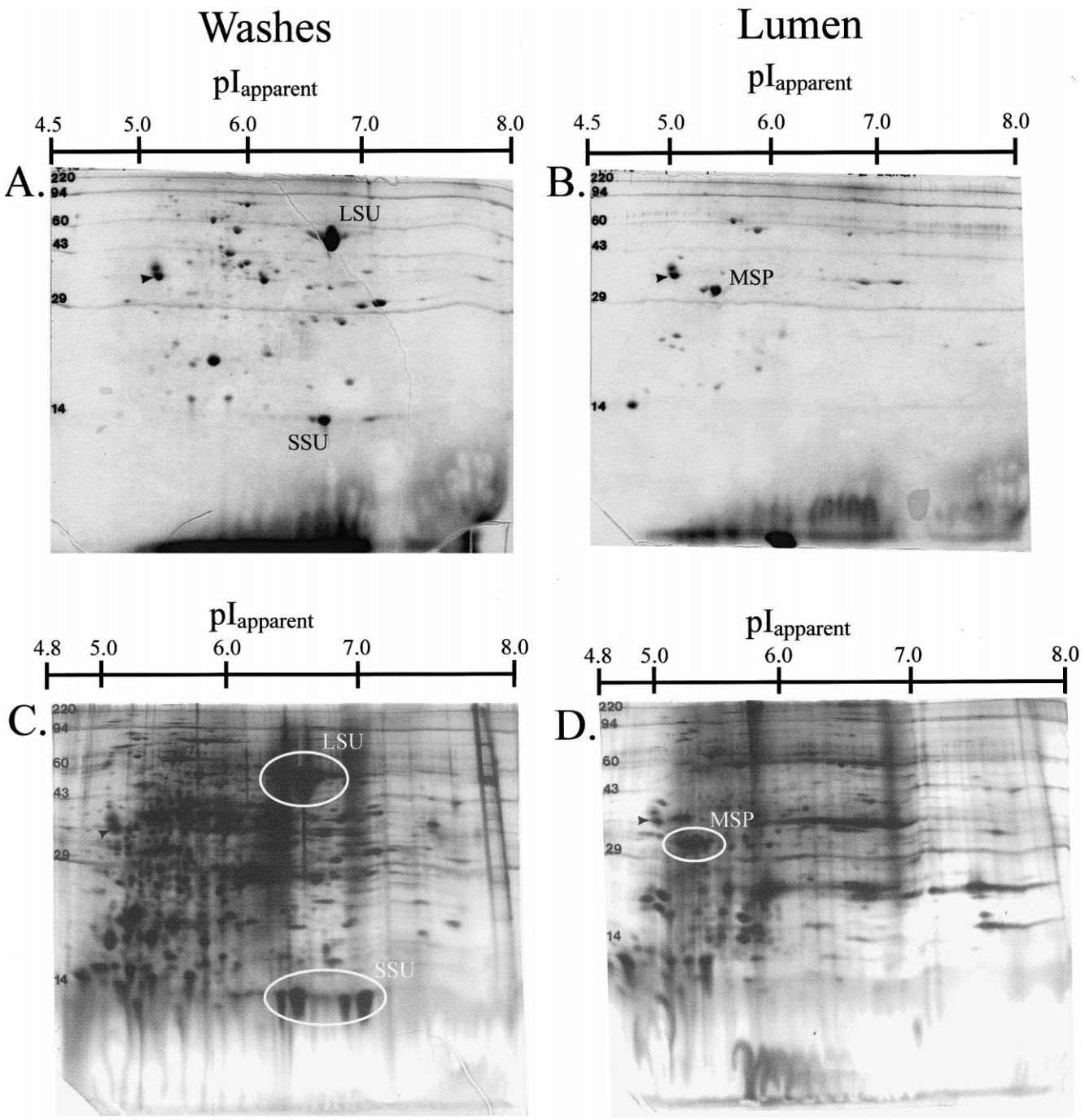

Fig. 4. 2D separation of washes and lumenal fractions. (A) and (C) are separations of the pooled washes stained with Coomassie blue and silver, respectively. (B) and (D) are separations of the lumenal fraction stained with Coomassie blue and silver, respectively. The apparent p*I*s resolved in the first dimension are shown above each panel while the apparent masses of the standard proteins are shown to the left of each panel. Some proteins are identified and labeled: LSU, large subunit of RuBp carboxylase; SSU, small subunit of RuBp carboxylase; MSP, photosystem II manganese stabilizing protein. An arrowhead indicates the location of the lower mass isoform of tropomyosin, which migrates at an apparent p*I* of 5.2.

ponents. Additionally, using modifications of the procedures presented in this communication, we have isolated a protein fraction enriched in lumenal proteins from the cyanobacterium *Synechocystis* 6803. We hope that the availability of a simple procedure for the isolation of the lumenal proteins of the chloroplast thylakoids will stimulate investigations of the properties and functions of the proteins contained within this subcellular domain.

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