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Title

Comparison of the α and β isomeric forms of the detergent n-dodecyl-D-maltoside for solubilizing photosynthetic complexes from pea thylakoid membranes.

Authors names and affiliation

Cristina Pagliano[a,*], Simone Barera[a],Fabiana Chimirri[a],Guido Saracco[a], James Barber[a,b,*]

[a]Department of Materials Science and Chemical Engineering - BioSolar Lab, Politecnico di Torino, Viale T. Michel 5, 15121 Alessandria, Italy
[b]Division of Molecular Biosciences, Department of Life Sciences,Imperial College London, London SW7 2AZ, United Kingdom

Corresponding authors

[*]Corresponding authors. E-mail: cristina.pagliano@polito.it; Tel. No. +39 131 229301; Fax No. +39 131 229344;and E-mail: j.barber@imperial.ac.uk; Tel. No. +44 207 594 5266; Fax. No. +44 207 594 5267

Abstract

Mild non-ionic detergents are indispensable in the isolation of intact integral membrane proteins and protein-complexes from biological membranes. Dodecylmaltoside (DM) belongs to this class of detergents being a glucoside-based surfactant with a bulky hydrophilic headgroup composed of two sugar rings and a non-charged alkyl glycoside chain. Two isomers of this molecule exist, differing only in the configuration of the alkyl chain around the anomeric centre of the carbohydrate head group, axial in α-DM and equatorial in β-DM. In this paper, we have investigated the solubilizing properties of α-DM and β-DM on the isolation of photosynthetic complexes from pea thylakoids membranes maintaining their native architecture of stacked grana and stroma lamellae. Exposure of these stacked thylakoids to a single step treatment with increasing concentrations (5-100 mM) of α-DM or β-DM resulted in a quick partial or complete solubilization of the membranes. Regardless of the isomeric form used: 1) at the lowest DM concentrations only a partial solubilization of thylakoids was achieved, giving rise to the release of mainly small protein complexes mixed with membrane fragments enriched in PSI from stroma lamellae; 2) at concentrations above 30 mM a complete solubilization occurred with the further release of high molecular weight protein complexes identified as dimeric PSII, PSI-LHCI and PSII-LHCII supercomplexes. However, at concentrations of detergent which fully solubilizedthe thylakoids, the α and β isomeric forms of DM exerted a somewhat different solubilizing effect on the membranes: higher abundance of larger sized PSII-LHCII supercomplexes retaining a higher proportion of LHCII and lower amounts of PSI-LHCI intermediates were observed in α-DM treated membranes, reflecting the mildness of α-DM compared with its isomer.

Keywords: n-dodecyl-α-D-maltoside; n-dodecyl-β-D-maltoside; thylakoids; membranes solubilization

Abbreviations:

ATP-ase Adenosine triphosphate synthase; BN-PAGE Blue native polyacrylamide gel electrophoresis; Chl chlorophyll;CMC critical micelle concentration; Cyt cytochrome; DGDG digalactosyldiacylglycerol; α-DM n-dodecyl-α-D-maltoside; β-DM n-dodecyl-β-D-maltoside; HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC High performance liquid chromatography; LHC Light harvesting complex; MES 2-(N-Morpholino)ethanesulfonic acid; MGDG monogalactosyldiacylglycerol;PG phosphatidylglycerol; PS Photosystem; RC Reaction centre; SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SQDG sulfoquinovosyldiacylglycerol

1. Introduction

Detergents are indispensable in the isolation of integral membrane proteins and protein-complexes from biological membranes to study their intrinsic structural and functional properties [1–5]. However for membrane protein structure/function analysesincluding crystallization, the demands on sample preparation are complex. The challenge is to obtain complete extraction and separation of different proteins species from the membrane while maintaining the structure and function of the proteins or holocomplexes in a native state. Therefore for an efficient solubilization, the membrane characteristics, the nature of the protein and the nature of the detergent have to be considered.

Thylakoid membranes in cyanobacteria and in chloroplasts of green algae and higher plants are the site of primary photosynthetic reactions, where electrons are transferred through a series of photosynthetic redox active protein complexes to convert light energy into biologically useful chemical energy. The lipids that constitute thylakoid membranes show a unique composition compared with other biological membranes [6,7]. Specifically, thylakoid membranes have high proportions of the glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG). They also have low proportions of the phospholipid phosphatidylglycerol (PG).

Embedded in this lipid bilayer there are four multiprotein complexes which act together to carry out oxygenic photosynthesis: photosystem I (PSI), photosystem II (PSII), ATP-synthase (ATP-ase) and cytochrome *b6/f* complex (Cyt *b6/f*), consisting mostly of hydrophobic membrane integral α-helical protein subunits. Included in these subunits are also the light harvesting complexes of PSI and PSII (LHCI and LHCII). Alfa-helical transmembrane proteins are usually found with one to twelve transmembrane helices or with a lipid anchor [8] and have a complex pattern of hydrophobic interactions with the membranes lipid bilayer. It is theamphipathic nature of these interactions which enables the detergent induced solubilization of the protein-lipid assembly in aqueous solutions. Moreover, in many cases the activity/intactness of solubilized protein complexes has been shown to be dependent on the co-extraction of lipids with the proteins [9]. Biochemical and molecular biological analyses have highlighted the importance of the presence of particular lipids within various protein complexes of the plant, algal and cyanobacterial thylakoid membranes, either for their functionality or structural integrity. Taking as an example the PG molecule, it was demonstrated to play a key role in the proper functionality and structural integrity of photosynthetic complexes. Depleting thylakoid membranes of PG by treatment with phospholipases resulted in the suppression of the PSII activity, demonstrating the functional importance of this lipid in the electron transport at the Q_B-binding site in PSII complexes [10–12]. Moreover, PG was found to be necessary for the assembly and stabilization of the photosynthetic complexes, because its presence enhanced the formation of dimeric PSII and trimeric PSI complexes [13–16]. The intimate interaction of thylakoid lipids with photosynthetic complexes has been revealed by crystallography where one molecule of MGDG and three molecules of PG were assigned within the crystal structure of the PSI complex [17], while a total of 20 lipid molecules (6 of MGDG, 5 of DGDG, 5 of PG and 4 of SQDG) were modeled in each monomer of the crystal structure of the PSII complex [18]. From these studies and others, it becomes evident that it is important not to over-solubilize the thylakoid membranes with extensive detergent treatments if functional and structural integrity is to be maintained for the isolated photosynthetic complexes.

In general, there are different classes of detergents which can be used for solubilizing membrane proteins. Among the most commonly used are the non-ionic surfactants such as the polyoxyethyleneglycole and the alkylglucosides. They are considered “mild” detergents since they mainly solubilizemembrane proteins by breaking lipid-lipid and lipid-protein interactions rather than protein-protein interactions, leaving the structure of the isolated protein or protein complex intact. Moreover, detergents from one homologous series with longer alkyl chain are generally milder than the ones with short alkyl chains and also, the larger the headgroup the milder the detergent [19,20]. Dodecylmaltoside (DM) belongs to the glucoside-based non-ionic surfactants with an extremely large and rather stiff hydrophilic headgroup made up of two sugar rings (maltose) and a non-charged alkyl glycoside chain (C12). Depending on the configuration of the alkyl chain around the anomeric centre of the carbohydrate head group, two isomers of this molecule are distinguishable: α-DM and β-DM, which have identical hydrophilic-lipophilic balances but significant differences in molecular architecture, because the C12 chain is respectively in the axial or equatorial orientation with respect to the polar head. Some of the main chemical features of the two DM isomers, based on published studies [21–23], are summarized in Figure 1.

Between the two isomers, β-DM has been extensively used in solubilization of integral membrane proteins with retention of their functional properties [e.g., 24–28], including isolation from higher plants thylakoids of multi-subunits photosynthetic complexes [e.g., 29–34], which in some cases has led to crystal structures for PSI [35] and LHCII [36,37]. Similarly, the determination of crystal structures of PSI and PSII from cyanobacteria has also been achieved using β-DM [17,18,38,39].

In recent years attention has turned to using the α-isomer of DM to solubilize plant thylakoid membranes in a single step treatment [40], obtaining either intact grana [41] or fully solubilized PSI and PSII complexes [42–44]. However, until now no crystal structures of photosynthetic membrane protein complexes isolated with α-DM, generally perceived to be milder than β-DM, have been reported.

For the correct use of detergents it is necessary to have an idea of how and in which amounts, they interact with integral membrane proteins and membrane lipids. An important factor for membrane solubilization is the critical micelle concentration (CMC) of the detergent. Detergents are soluble in aqueous solutions; however, when the concentration of detergent in solution increases, self-association of the single detergent molecules leads to a sharp phase shift and micelles start to form at the CMC [45]. Concentrations of detergent above the CMC are able to sustain a solubilization of hydrophobic and amphipathic molecules [45]. With this in mind, in this paper we have carried out a careful analysis of the action of the α-DM and β-DM on thylakoid membranes of the higher plant *Pisum sativum*. The approach adopted to study the solubilizing properties of the two isomeric forms of the same detergent molecule at increasing concentrations, combined with detailed biochemical analyses of each solubilized fraction, is important in order to understand the overall solubilizing action of each form of the detergent on the isolation of differently sized membrane protein complexes. Comparison of the action of the two isomeric forms of DM was achieved by the separation of solubilized membrane protein complexes with blue native polyacrylamide gel electrophoresis (BN-PAGE) and size-exclusion chromatography to preserve their native organization, followed by the application of denaturing SDS-PAGE to determine their polypeptide compositions. In this way it was possible to compare the two isomeric forms of DM and identify the optimal concentrations for their use (at a fixed lipid/Chl ratio and protein/Chl ratio of the same initial thylakoids) for the isolation of thylakoid protein complexes, with particular focus on the isolation of PSII complexes.

2. Material and methods

2.1. Plant growth conditions

Before sowing, pea (*Pisum sativum* L., var. Palladio nano) seeds were treated as described in [46]. Germinated seedlings were transferred to pots and grown hydroponically in Long Ashton nutrient solution [47] in a growth chamber with 8 h daylight, 20°C, 60% humidity and 150 µmol m^{-2} s^{-1} photons. Leaves from plants grown for 3 weeks were harvested and used for experiments.

2.2. Isolation of thylakoid membranes

Thylakoid membranes from 21 day-old pea leaves wereisolated so as to maintain their native granal and stromal lamellae organization, referred to as stacked thylakoids from here on. Briefly, pea leaves were disrupted by grinding with a blender in 50 mM HEPES pH 7.5, 300 mM sucrose and 5 mM $MgCl_2$. The suspension was passed through a filter and the filtrate was centrifuged at 1,500 x*g* for 10 min. The pellet was washed once by centrifugation in the same buffer and then homogenized in 5 mM $MgCl_2$ and diluted 1:1 with 50 mM MES pH 6.0, 400 mM sucrose, 15 mM NaCl and 5 mM $MgCl_2$ followed by 10 min centrifugation at 3,000x*g*. The resulting pellet of thylakoid membranes was washed once by centrifugation in 25 mM MES pH 6.0, 10 mM NaCl and 5 mM $MgCl_2$. Thylakoid membranes were suspended and stored in 25 mM MES pH 6.0, 10 mM NaCl, 5 mM $MgCl_2$and 2 M glycine betaine(MNMβ buffer).

2.3. Thylakoids solubilization withα-DM and β-DM

Equal amounts of stacked thylakoid membraneswere suspended in MNMβ buffer and solubilizedat a Chl concentration of 1 mg $ml^{-1}$with different concentrations (ranging from 5 to 100 mM) of α-DM orβ-DM in the dark on ice for 1 min. Non-solubilized material was then removed by double centrifugation at 21,000 x*g* for 10 min at4°C.For each concentration of detergent, membrane pelletswere resuspended in MNMβ buffer and,together with their corresponding supernatants, quantified on a Chl basis. Finally the percentage of solubilization for eachdetergent treatment was calculated as the ratio between the amount of Chl in the supernatant over the total amount of Chl of thylakoids treated with the detergent.

2.4. Spectroscopic measurements

Absorption spectra were recorded using a Lambda25 spectrophotometer (Perkin Elmer). The Chl *a*and *b* contentof solubilized thylakoids were measured after extraction in 80% acetone using extinction coefficients given by Arnon [48].

2.5. Biochemical characterization of solubilized membranes

The protein composition of supernatants wasinvestigated by native non-denaturing analyses (i.e., BN-PAGE and size-exclusion chromatography) and byelectrophoresis under denaturing conditions (SDS-PAGE).

Protein complexes from solubilized thylakoids were separated byBN-PAGE system according to Schagger and von Jagow[49]using a linear gradient gel (3-12% acrylamide). For molecular mass markers, a mixture of lyophilized standard proteins (Amersham, high molecular weight, GE Healthcare) was used.For the second dimension, the BN-PAGE lanes were cut out and denatured in a buffer made of66 mM Na_2CO_3, 2%(w/v) SDS and 0.66%(v/v) 2-mercaptoethanol at 25°C for 30 min and subjected to SDS-PAGE on 15%polyacrylamide gel containing6 M urea using the Laemmli's system [50]. The same electrophoretic system was used for mono-dimensional SDS-PAGE, with the only modification being the lowering of polyacrylamide to 12.5%.Pre-stained protein size markers (Bio-Rad, Precision Plus) were used for estimation of apparent size of thylakoids components. The separated proteins were visualized by Coomassie brilliant blue R-250 or silver staining.

The same supernatants used for electrophoresis were subjected to size-exclusionchromatography on a Jasco HPLC system with aBioSep-SEC-S 3000 (Phenomenex) column. The 20-µlsamples injected contained 6 µg Chl and the profiles weremonitored at 400 nm. The mobile phase consisting of 20 mM MES pH 6.5, 10 mM $MgCl_2$, 30 mM $CaCl_2$, 0.5 Mmannitol and 0.59 mM α-DM or β-DM passed through the column at aflow rate of 0.5 ml min^{-1}. To collect peaks, the flow rate of the mobile phase through the column was reduced to 0.2 ml min^{-1}, and after the appearance of the first green material,fractions of 0.4 ml each were collected. Fractions containing peaks were kept on ice and analyzed within one hour by absorption spectroscopy and loaded on SDS-PAGE.

3. Results

3.1. Degree of thylakoid membrane solubilization byα-DM and β-DM andpigment and protein composition of the solubilized fractions

Stacked pea thylakoid membranes at a Chl concentration of 1 mg ml^{-1}were solubilized in a single step with 5, 10, 20, 30, 50, 70 and 100 mM α-DM or β-DM for 1 min at 4°C.After this short detergent treatment, unsolubilized thylakoids were spun down by centrifugation at 21,000 x*g*. Hereafter we call "solubilized thylakoids" the mixture of fully solubilized protein complexes and small membrane fragments that at this speed remain in the supernatant, whereas largest membrane patches sediment.

The efficiency of α-DM and β-DM for thylakoid solubilization was evaluated as the ratio between the amount of Chl present in the supernatant after centrifugation over the total amount of Chl of thylakoids treated (1 mg Chl used as starting material) at each detergent concentration. Starting from thylakoids characterized by a Chl *a/b* ratio of 3.15 ±0.03 an almost full solubilization was achieved by using concentrations above 30 mM both for α-DM and β-DM (Fig. 2A), whose supernatants display Chl *a/b* ratios comparable with the values of the starting thylakoids (Fig. 2B). In contrast, at the two lowest concentrations of detergent (5 and 10 mM) onlya partial solubilization of thylakoids occurred (within 30% of the initial amount), with an efficiency slightly higher in membranes treated with α-DM than with β-DM. At these concentrations, whether for α-DM or β-DM, the supernatants consistedof a higher proportion of protein complexes containing Chl *a* rather than Chl *b,*as attested by Chl *a/b* ratios above 4 for the α-DM treated thylakoids and between 3.6 and 4 for the β-DM treated membranes.At 20 mM a higher yield of solubilization was found (above 80%) either with thylakoids treated with α-DM orwith β-DM. However, at this concentration the detergent, even if not fully solubilizing the

membranes, gave rise to supernatants with a pigment-protein composition similar to the starting thylakoids, as judged by having the same Chl *a/b* ratio (Fig. 2B).

The polypeptide composition of thylakoid membranes solubilized with α-DM and β-DM was then assessed by SDS-PAGE. As shown in Figure 3, at low concentrations (5 and 10 mM), regardless of the isomeric form of the detergent employed, the proportion of PSI in the supernatantswas higher than at all other concentrations of detergent tested, accounting for the higher Chl *a/b* ratios (Fig. 2B). Moreover, at these sub-optimal detergent concentrations the increased Chl *a/b* ratios displayed by thylakoids treated with α-DM when compared to membranes solubilized with β-DM are in agreement with the lower proportion of LHCII present in these supernatants derived from the α-isomer.In addition, the supernatants after 5 and 10 mM α-DM or β-DM treatment contained ATP-ase at levels higher than at all the other concentrations tested relative to the Chl level. In contrast, the SDS-PAGE showed no significant differences in the polypeptide composition of thylakoids solubilized at concentrations above 20 mM either with α-DM or β-DM, in agreement with the similarityin the Chl *a/b* ratios (Fig. 2B).

3.2. Typology and abundance of solubilized protein complexes analyzed by BN-PAGE

To make a detailed examination of the protein complexes solubilized from stacked thylakoids at a fixed Chl concentration (1 mg ml^{-1}) by a single step treatment with α-DM or β-DM at different concentrations, we analyzed each supernatant using two complementary assays retaining the native form of the solubilized protein complexes: BN-PAGE and size-exclusion chromatography.BN-PAGE is a special type of native electrophoresis for high-resolution separation of membrane protein complexes powerful in the range of molecular weight between 10 and 10.000 kDa [51]. Figures4A and 4B show respectively BN-PAGE profiles of thylakoids solubilized with different concentrations of α-DM and β-DM (Figures 4C and 4D are the same gels as above but Coomassie stained). At concentrations of detergent which fully solubilized the membranes (above 30 mM in both cases), several bands were detected. According to previous reports [52–54] and to the interpretation of the denaturing second dimensions profile of the BN-PAGEs of thylakoids solubilized with 70 mM α-DM (Fig. 5A) and 70 mM β-DM (Fig. 5B), these bands can be assigned from top to bottom of the gel as:

1) at a molecular masses of >669 kDa several bands consisting of PSII-LHCII supercomplexes;

2) at about 600 kDa an intensive green band consisting of PSI-LHCI co-migrating withPSII core dimerdue to their similar molecular mass;

3) at about 480 kDa a sharp band corresponding to PSIvisible mainly in β-DM treated membranes, likely representing transition states or disassembly states of PSI-LCHI complexes;

4) atabout300-250 kDa, three bands closely adjoined are due to ATP-ase (280-300 kDa), monomeric PSII core (around 280 kDa) and dimeric Cyt *b6/f* complex (around 250 kDa);

5) at about 140 kDa a green band containing LHCII subunits,probably in their trimericform;

6) at about 120 kDaa band corresponding to monomeric Cyt*b6/f* complex;

7) at about 70 kDa a band containing LHCII subunits, most likely in theirmonomeric form.

In the case of these native gels, bands corresponding to pigment-protein complexes with high molecular weights (i.e., PSII-LHCIIsupercomplexes) were detected only at concentrations of detergent which fully solubilized the membranes, and they were much more abundant in thylakoids solubilized with α-DM (Fig. 4A,

4C, 5A) than with β-DM (Fig. 4B, 4D, 5B).Moreover, at these detergent concentrations (30-100 mM) there was acommon profile between α-DM and β-DM in the range between 600-100 kDa, with thepresence of high amounts of PSI-LHCI co-migrating with dimeric PSII andhigh quantity of LHCII trimers. One noticeable difference, however, was that thylakoids treated with 30 mM gave a relatively higher level of monomeric PSII paralleled by a slightly lower abundance of PSII-LHCII supercomplexes, differing partially also in size with respect to those observed at the highest detergent concentrations tested. Another noticeable difference was the presence of a higher amount of LHCII in its monomeric form in thylakoids solubilized with β-DM (band at about 70 kDa in Fig. 4B, 4D) than in α-DM treated membranes, as also clearly shown by the second dimension analysis (Fig. 5B). Despite the loading of the BN-PAGE with the same amount of Chl for each detergent treatment (Fig. 4A-C and 4B-D, 20 μg Chl per lane), at the two lowest concentrations of α-DM and β-DM tested (5 and 10 mM)only some bands were detectedbetween 300-250 kDa (corresponding to ATP-ase, monomeric PSII and dimeric Cyt *b6/f* complex), whose total intensity was lower than the total intensity derived from the bands observed at higher DM concentrations.The discrepancy between the similar total proteinsstaining intensity ineachdetergent treatment displayed on SDS-PAGEs, both for α-DM and β-DM solubilized thylakoids (respectively Fig. 3A and 3B, 4 μg Chl per lane), with the lower total intensities of the corresponding counterparts on BN-PAGEs when low concentrations of detergents were used (respectively Fig. 4A-C and 4B-D), can be explained by the presence in supernatants of thylakoids treated with 5-10 mM DM of small membrane fragments mixed with fully solubilized protein complexes that, given their large size, do not enter into the pores of the native gels, remaining in the loading wells.Moreover, α-DM and β-DM when used at low concentrations (5 and 10 mM), exerted a somewhat different solubilizing action with the same thylakoids (Fig. 4A-C and Fig. 4B-D), as reflected in the higher abundance of PSI at about 480 kDa and LHCII trimers in β-DMtreated membranes with respect to α-DM counterparts. Nevertheless,taking into account that at these low concentrations of detergent the Chl *a/b* ratios were higher in α-DM treated membranes (Fig. 2B),where the LHCII were less abundant (Fig. 3A) than in supernatants of thylakoids solubilized with β-DM, it is likely that big fragments of membranes produced by α-DM contain more PSI than fragmentsgenerated by β-DM.When compared to the lowest concentrations, at 20 mM with both detergents more bands were detected, but of less intensity than the corresponding ones obtained with concentrations above 30 mM, denoting an incomplete solubilization of the membranes especially in terms of large-sized protein complexes, as also evidenced by the presence of several faint bands (mainly observed with the α-DM treated membranes) in the region of the PSII-LHCII supercomplexes instead of well-defined bands found at concentrations of detergent fully solubilizing the thylakoids.

3.3. Typology and abundance of solubilized membrane fragments and protein complexes analyzed by size-exclusion chromatography

The results obtained by BN-PAGE suggested that thylakoids solubilized at low concentrations of detergent contain some fully solubilized protein complexes mixed with small membrane fragments that, because of their large size, do not enter into the pores of the native gels.However, we found that the size of the small membrane fragments included in the mixture of solubilized components was no larger than about 0.22 μm, the size of the filter through which the “solubilized” material was passed before the injection into the size-exclusion column.

The same amount of Chl (6 µg) for each supernatant used for electrophoresis was subjected to size-exclusion chromatography. Regardless of the isomeric form used, among the different detergent treatments a similar profile was found withthylakoids solubilized with 5 and 10 mM, and with those treated with 30-100 mM, whereas membranes subjected to 20 mM DM showed an exclusive profile different from the other two groups. In figure 6the size-exclusion chromatograms of representatives of these three groups of solubilized thylakoids (5, 20 and 70 mM) are shown for α-DM (A) and β-DM (B), where their peaks are numbered within each detergent panel in a sequential order according to their increasing retention times. Data are summarized in Table 1 along with their corresponding intensities.For both detergents, fractions containing peaks were collected after the appearance of the first green material and analyzed by absorption spectroscopy (Fig. 7A-B) and SDS-PAGE (Fig.7C).Figure 6 shows that treatment with detergent concentrations which do not fully solubilize thylakoids (5 and 20 mM) resulted in rather different size-exclusion chromatographic profiles when comparing α-DM (Fig. 6A) with β-DM treated membranes (Fig. 6B). These differences became less evident at 70 mM DM, where a complete solubilization occurs (Fig. 2A).

As can be seen in Fig. 6, for both detergents the maximum number of distinguishable peaks (or shoulders) was five, each one characterized by retention times (rT) generally rather similar and stable among the different concentrations of each detergent, especiallyin the case of β-DM treated membranes (Table 1).The following assignment of each peak is based on:

1)the spectroscopic characteristics of fractions contained within each peak (absorption spectrasee Fig. 7 A-B;for maximum absorption in the Qy absorption region of the Chl, *A*480/*A*410 ratio (indicative of Chl *b* level) and *A*700/*A*670 ratio (indicative of PSI level) see Table 2) and their corresponding SDS-PAGE profiles (see Fig. 7C);

2) the correspondence between the number and intensity of peaks on the chromatogram at a certain concentration of detergent and the corresponding number and intensity of bands of pigment-protein complexes separated on the corresponding lanes of the BN-PAGEs(see Fig. 4).

Based on these considerations, we attribute the first peak eluting between8.59-9.16 min (rT1, Table 1) to patches of membranes containing a large proportion of PSI together with some LHCII trimers and ATP-ase, likely representing pieces of solubilized stromal lamellae and grana margins. This conclusion is based on absorption spectra (Fig. 7A-B) peaking at 679.9 and 679.1 respectively in α-DM and β-DM fractions (Table 2), their similar moderately high *A*480/*A*410 ratios (0.69-0.70) and high*A*700/*A*670 values (0.26-0.28), theirelectrophoretic profiles showing the presence of PSI, LHCII and ATP-ase subunits (lane 1, Fig. 7C)and by taking into account previous results from BN-PAGEs. This peak was more intense mainly when thylakoids were treated with not fully solubilizing concentrations of detergent (5-20 mM) and was more evident with α-DM treated thylakoids. Moreover, with α-DM only, this peak was also detected with fully solubilizing concentrations (70 mM).

The second peakwith a very similar retention time (rT2) between α-DM and β-DM treated membranes, ranging between 12.10-12.39 min (Table 1),appeared as an intense peak in α-DMtreated thylakoids only at concentrations of detergent fully solubilizing the membranes (70 mM, Fig. 6A). We assign this peak to PSI-LHCI complexes co-eluting with dimeric PSII cores. This conclusion is in agreement with previous results obtained by BN-PAGE and is based on the absorption spectra peaking at 679.3 and 679.4 respectively in α-DM and β-DM fractions (Table 2), their similar rather low *A*480/*A*410 ratio (0.50) and high *A*700/*A*670 values (0.21-0.23), and their electrophoretic profiles showing both PSI-LHCI and PSII subunits (lane 2, Fig. 7C).

The third peak,detectable only at low detergent concentrations, as a distinct rather wide peak in thylakoids solubilized withα-DM only at 5 mM (Fig. 6A) and as a very narrow peak closely adjoined with the second and fourth peak in membranes treated with 5 and 20 mM β-DM (Fig. 6B), showed two different retention times (rT3) at 14.56 min in thylakoids solubilized with α-DM and between 13.44-13.51 min in β-DM treated membranes (Table 1). Their electrophoretic profiles showed predominantly PSII subunits (lane 3, Fig. 7C), even if in the case of β-DM there was a higher contamination mainly by PSI components and partially by LHCII, due to the difficulties in obtaining clean fractions of the third peak, because its physical proximity with adjacent peaks. This is also reflected in the quite different absorption spectra peaking in α-DM treated membranes at 674.3, typical of a pure plant PSII, and at 676.7 in the case of β-DM fractions(Table 2) and also in the higher *A*480/*A*410 and *A*700/*A*670 ratios displayed by β-DM treated thylakoids, in agreement with results from BN-PAGE.

The fourth peak was constantly present at all the concentrations of α-DM and β-DM tested, with a retention time (rT4) rather constant between 14.53-14.95 min, with the only exception of the 5 mM α-DM treated thylakoids (Table 1), where it generates a shoulder due to its low quantity.This peak is attributed to LHCII complexes based on absorption spectra peaking at 675.1 and 675.2 respectively in α-DM and β-DM fractions (Table 2), the very high *A*480/*A*410 ratios(1-0.94) and extremely low *A*700/*A*670 values (0.04-0.06). Moreover, their electrophoretic profiles show an enrichment in LHCII subunits (lane 4, Fig. 7C) and from BN-PAGE the trimericLHCII form dominates.

Finally, the fifth peak is clearly detectable only at the lowest detergent concentration in thylakoids solubilized with α-DM (Fig. 6A) with a retention time (rT5) of 17.07 min (Table 1), whereas in thylakoids solubilized with β-DM it is detected at all concentrations tested, with a different and very stable retention time (15.71-15.74 min). From the comparison of absorption spectra of α-DM and β-DM fractions contained within the fifth peak (Fig. 7A-B), it is evidentthat there is a difference in the Chl *b* level, being more abundant in β-DM treated thylakoidsas attested by their higher *A*480/*A*410 ratio (Table 2) and the presence of a higher proportion of LHCII subunits (lane 5, Fig. 7C) with respect to α-DM treated membranes. When compared to the fourth peak, the fifth peak in β-DM treated membraneson SDS-PAGE showed a higher proportion of monomeric subunits of LHCII.This observation, and taking into account results from BN-PAGE where a higher quantity of LHCII monomers and a lower proportion of PSII-LHCII supercomplexes were detected in β-DM treated membraneswith respect to thylakoidstreated with α-DM, suggests that β-DM is more effective at detaching LHCII monomers from the supercomplexes than its isomer.

4. Discussion

In this paper we have compared in detail the different solubilizing actions of the two isomeric forms of DM using a wide range of concentrations and identical thylakoids (same lipid/Chland protein/Chl ratios) maintaining their grana and stromal lamellae regions, with the aim to establish the best conditions to isolate fully solubilized protein complexes still intact. The main differences found in the solubilization at different concentrations of α-DM and β-DM are discussed in relation to the physical localization of the isolated photosynthetic complexes in different regions of the thylakoid membrane. The discussion takes into account the interaction of detergents with membrane proteins and lipids and the structural differences between the α and β isomeric forms of DM.

By using low concentrations (5-10 mM) of DM, with both isomers we obtained only a partial solubilization of thylakoids reaching a maximum of 30% of the initial membranes (Fig. 2A). The solubilized fraction isolated by centrifugation consisted of:1) small membrane fragments enriched in PSI, not entering in the native gels (Fig. 4) and identified by size-exclusion chromatography as peak 1 (Figs. 6-7) and 2) stromalproteins (ATP-ase, PSI, LHCI) and other integral membrane protein complexes (monomeric PSII, Cyt *b6/f* both dimer and monomer and LHCII). At these low concentrations of DM, despite a general common pattern of solubilization between the two isomers, it was clear that β-DM generated more PSI than α-DM (Fig. 6B). From these results, weconcludethat under the conditions employed proteins mainly localized in the stromal lamellae were solubilized whereas unsolubilized material and stacked grana lamellae were spun down in the pellet. This is consistent with the lateral heterogeneity modelfor the distribution of pigment-protein complexes of the thylakoid membranes of Andersson and Anderson [55] and in accordance with recent findings ofMorosinotto et al. [41], where a Chl/DM ratio below 1:12 was used to isolate structurally intact grana membranes from different higher plants thylakoids.

At 20 mM with both DM isomers we obtained a higher yield of thylakoid solubilization, reaching values above 80% of the initial membranes (Fig. 2A). If compared to the lowest concentrations tested (5 and 10mM), the supernatant derived from the 20 mM DM treatment contained: 1) a significantly lower amount of small membrane fragments detected by size-exclusion chromatography as peak 1 (Fig. 6), with more protein complexes entering the native gels (Fig. 4A,C); and 2) a higher amount of medium sized integral membrane protein complexes fully solubilized as PSII dimers and PSI-LHCI supercomplexes (Fig. 4) plus smaller complexes also found at lower thylakoids Chl/DM ratios. Comparing the two isomers, some differences were found: the level of small membrane fragments was higher with α-DM, whereas the proportion of fully solubilized PSI-LHCI complexes and their intermediates was higher with β-DM treated thylakoids(Fig. 4 and Fig. 6). From these results, we suggest that at this Chl/DM ratio (1:20) protein complexes localized in the stroma lamellae and also grana margins/margin ends were subjected to the detergent action, in accordance with findings by van Roon et al. [40] and Dekker et al. [42], where a thylakoids Chl/DM ratio around 1:18 was used to isolate grana in a relatively intact state as paired membranes with all other thylakoid components solubilized.

Whenusing 30mM and higher concentrationswith both DM isomers, we obtained an almost total solubilization of thylakoids (Fig. 2A). Consequently high molecular weight pigment-protein complexes of PSII dimers, PSI-LHCI and PSII-LHCII supercomplexes were isolated,the level of which increased at the highest concentrations of detergent used (Fig. 4).Due to the predominant localization of PSII dimers and PSII-LHCII supercomplexes in grana stacked thylakoids [55,56], we can conclude that atChl/DM ratio of 1:30 and above the detergents can access the partition regions and solubilize large pigment-protein complexes localized in grana lamellae. This conclusion is consistentwith previous results of Eshaghi et al. [30], where β-DM was used at a Chl/DM ratio of 1:40 to isolate PSII-LHCII supercomplexes directly from spinach thylakoids. It is also in linewith the findings of Morosinotto et al. [43] who isolated PSII-LHCII supercomplexes from thylakoids of a PSI-less barley mutant in a single step solubilization with α-DM using a Chl/DM ratio of about 1:24, a value comparable to 1:40 when taking into account that PSI was absent.

In summary, our study has revealed that, using a short time single step solubilization of the thylakoid membranes, it is possible to isolate in different amounts fully solubilized ATP-ase, Cyt *b6/f*, PSI and PSII complexes either in protomeric or different self-associated states depending on the type of detergent used

(α-DM or β-DM) and its concentration (between 5-100 mM). This experimental evidence corroborates the idea that the choice of the surfactant and its concentration can considerably influence the abundance and oligomeric state in which protein complexes can be isolated from the thylakoid membrane. We can attempt to explain this phenomenon by taking into account the interaction of detergent molecules with membrane lipids and proteins.

A number of investigations have dealt with the interaction of detergents with pure phospholipids. These studies have led to the formulation of the three stage model for detergent-lipid-protein interactions [3]. Stage I and III of the model describe the starting and end points of solubilization. In stage I, detergent molecules are incorporated into the phospholipid-bilayer, while in stage III, all lipids are solubilized in detergent micelles. In stage II, detergent molecules incorporated in lipid bilayers coexist with lipid molecules incorporated in detergent micelles. Due to a thermodynamic equilibrium between both the solubilized and non-solubilized phases, the concentration of free detergent is kept constant and close to the critical micellar concentration (CMC). Interestingly, it was shown that if the pure phospholipid bilayer was replaced by a more complex system, the concentration of free detergent increased with progressive solubilization, indicating that lipid solubilization is influenced by the lipid composition of the membrane [5]. The fact that thylakoid membranes of plants have a low proportion of the phospholipid PG and a high proportion of the glycolipids MGDG, DGDG and SQDG [6,7] and that they are one of the most densely packed biological membranes (in grana 75% of the membrane area is occupied by proteins) [57,58] does not provide a simple membrane system to explain the action of detergents like DM. Nevertheless, we can assume that in the first stage detergent is taken up in a non-micellar form, predominantly by the lipid phase. At some point this is followed bydetergent-detergent interactions leading to destabilization of bilayer structure and membrane fragmentation, occurring in our conditions at the lowest concentrations of DM tested (5-10 mM). Further detergent addition leads to the formation of mixed-lipid-detergent micelles, exposures of the membrane proteins to micellar detergent and protein solubilization. In practice, the transitions between these different forms are usually not sharp, but overlapping, because the destabilization depends on the lipid species and the physical properties of the protein (e.g., its dimension) in the membrane, which produces some disorder in the organization of the lipid molecules allowing an easier access of the detergent to cooperatively interact with the protein to be solubilized. In this paper we demonstrated that the bigger is the protein to be solubilized the higher is the amount of surfactant necessary for its solubilization. This evidence is in agreement with findings by Morrissey et al. [59] where a progressive isolation from spinach thylakoids of solubilized proteins with increasing molecular weights was obtained by using increasing concentrations of Triton X-100.

In this paper some important differences in the solubilizing action of α-DM and β-DM became apparent. At concentrations of detergent which fully solubilized thylakoids, a higher abundance of larger sized PSII-LHCII supercomplexes retaining a higher proportion of LHCII (Fig. 4 and 5) and lower amounts of PSI-LHCI intermediates (Fig. 4) were observed with α-DM treated membranes. These differences can be explained by the different interaction/penetration into the membranes of the two isomeric forms of the DM, due to the different orientation of the alkyl chain with respect to the polar head (axial in α-DM and equatorial in β-DM). This structural difference changes their phase behaviors in dilute aqueous solutions (i.e., different micellar shape, spherical in α-DM and oblate ellipsoid in β-DM; lower CMC and lower aggregation number for α-DM than for β-DM) [21–23].

In conclusion, we have demonstrated that the two isomeric forms of DM exerted a somewhat different solubilizing effect on the thylakoid membranes and that, when compared at the same thylakoids Chl/DM ratio, α-DM is a milder detergent than β-DM,preserving the integrity even of the largest pigment-protein supercomplexes embedded into the thylakoids membranes.

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Figures and tables captions

Fig. 1.
Structural formula of n-dodecyl-α-D-maltoside and n-dodecyl-β-D-maltoside and chemical details of the two isomers.

Fig.2.
A.Degree of thylakoids solubilization with increasing concentrations of α-DM and β-DM. **B.** Chl *a/b* ratios of thylakoids solubilized with different concentrations of α-DM and β-DM, where Chl *a/b* ratio of starting thylakoids is 3.15 ± 0.03. Chlorophyll concentrations and Chl *a/b* ratios were determined in 80% acetone using the extinction coefficients reported by Arnon [48]. Each value is the average of at least ten independent determinations.

Fig.3.
Coomassie stained SDS-PAGEs of pea thylakoids solubilized with increasing concentrations of α-DM (A) and β-DM (B). Lane 1, prestained protein markers (Bio-Rad) with their apparent molecular weight (kDa) indicated on the left; lane 2, pea thylakoids (4 µg Chl); lanes 3-9, pea thylakoids (4 µg Chl) solubilized with different concentrations of detergent.

Fig. 4.
A-B. BN-PAGEs of pea thylakoids solubilized with increasing concentrations of α-DM (A) and β-DM (B). Lane 1, native high molecular weight marker (GE Healthcare);lanes 2-8, pea thylakoids (20 µg Chl) solubilized with increasing concentrations of detergent. Isolated complexes and subcomplexes were indexed as follows: supercomplexes (SC), PSI (I), PSII (II), LHCI (LI), LHCII (LII), ATP-ase (IV), Cyt *b6/f* complex (V). Arabic numbers were added in brackets to indicate the oligomeric status of the protein complex as, trimeric (3), dimeric (2) and monomeric (1).**C-D.** BN-PAGEs shown in A and B respectively Coomassie stained.

Fig. 5.
Second dimension SDS-PAGEs. The BN-PAGEs of stacked thylakoids solubilized with 70 mM α-DM (A) and 70 mM β-DM (B) were subjected to SDS-PAGE. The second dimensions were silver stained. Labels on the left of the SDS-PAGEs indicate the molecular weight positions, symbols above indicate the correspondence between thylakoids protein complexes (indexed as in Fig. 4) in the first dimension and their polypeptide composition in the second dimension.

Fig.6.
Size-exclusion profiles recorded at 400 nm of pea thylakoids solubilized with 5, 20 and 70 mM α-DM (A) and β-DM (B). 6 µg Chl were applied for each injection of thylakoids solubilized with 5, 20 and 70 mM DM. Peaks

are numbered in a sequential order according to their retention times on the three different spectra within each detergent panel.

Fig.7.

Spectroscopic and electrophoretic characteristics of fractions containing peaks. **A-B.**Room temperature absorption spectra of fractions containing peaks 1-5 from the size exclusion chromatography (Fig. 6) of thylakoids solubilized with α-DM (A) and β-DM (B). **C.** Identification of polypeptides present in each peak by SDS-PAGE. On the left, prestained protein markers (Bio-Rad) with their apparent molecular weight (kDa) indicated; lanes 1-5, fractions containing peaks 1-5 (equal volumes of each fraction were loaded).

Table 1

Details of size-exclusion profiles shown in Fig. 6: retention times (rT, min) and corresponding intensities (I, mV) of peaks (p) or shoulders (s) obtained during the elution of thylakoids solubilized with α-DM and β-DM.

Table 2

Spectroscopic details of the fractions containing peaks 1-5 from the size exclusion chromatography (Fig. 6). From the spectra shown in Fig. 7 the ʎ max (nm), representing the wavelength of the maximum absorption in the Qy absorption region of the chlorophylls, was determined and *A*480/*A*410 and *A*700/*A*670 ratios were calculated.

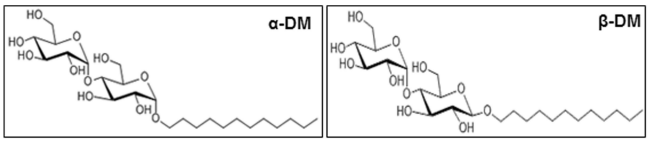
Table 1

	[DM]	rT1	I1	rT2	I2	rT3	I3	rT4	I4	rT5	I5
α-DM thylakoids	5 mM	8.59 (p)	27.673	12.10 (s)	≈4.299	14.56 (p)	18.255	15.95 (s)	14.828	17.07 (p)	17.881
	20 mM	8.84 (p)	50.567	12.39 (p)	11.304	-	-	14.95 (p)	43.025	-	-
	70 mM	9.16 (p)	3.750	12.30 (p)	87.437	-	-	14.90 (p)	81.279	-	-
β-DM thylakoids	5 mM	9.05 (p)	19.226	12.22 (p)	19.926	13.44 (p)	20.579	14.53 (p)	22.164	15.74 (p)	20.812
	20 mM	9.11 (p)	20.169	12.33 (p)	27.203	13.51 (p)	28.872	14.73 (p)	49.236	15.73 (p)	41.366
	70 mM	-	-	12.28 (p)	60.583	-	-	14.73 (p)	59.113	15.71 (p)	44.914

Table 2

	Peak	1	2	3	4	5
α-DM thylakoids	λ max (nm)	679.9	679.3	674.3	675.1	674.8
	*A*480/*A*410	0.69	0.50	0.39	1.00	0.58
	*A*700/*A*670	0.26	0.21	0.06	0.04	0.07
β-DM thylakoids	λ max (nm)	679.1	679.4	676.7	675.2	676.0
	*A*480/*A*410	0.70	0.50	0.49	0.94	0.94
	*A*700/*A*670	0.28	0.23	0.11	0.06	0.07

Figure 1



	α-DM	β-DM
Empirical formula	$C_{24}H_{46}O_{11}$	$C_{24}H_{46}O_{11}$
Partial molar volume (nm^3)	0.691	0.691
Chain length (nm)	1.8	1.8
Orientation chain/polar head	axial	equatorial
CMC (mol L^{-1})	$1.5 \cdot 10^{-4}$	$2 \cdot 10^{-4}$
Micellar shape	spherical	oblate ellipsoid
Micellar radius (Å)	24	34.4
Aggregation number	75	132

Figure 2

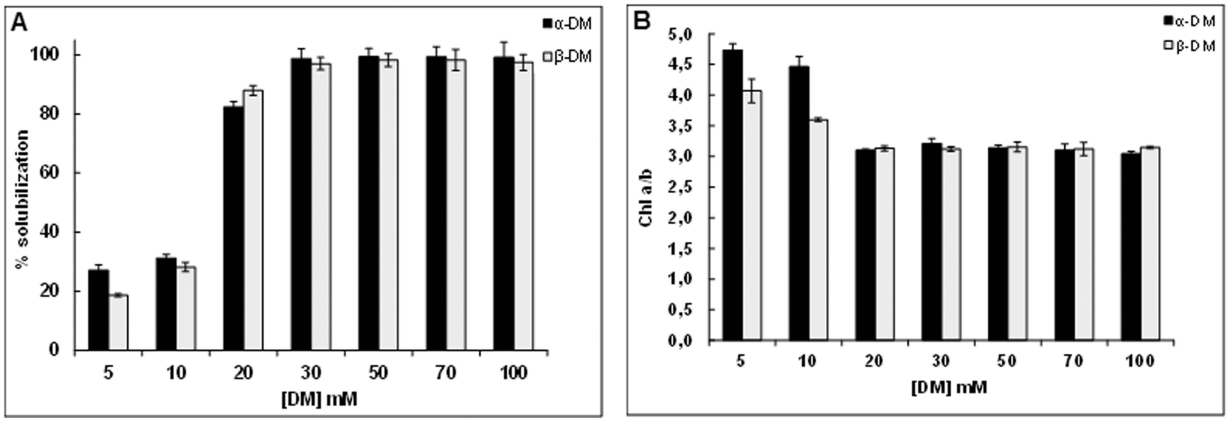

A
α-DM
β-DM
% solubilization
100
80
60
40
20
0
5
10
20
30
50
70
100
[DM] mM
B
α-DM
β-DM
Chl a/b
5,0
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
5
10
20
30
50
70
100
[DM] mM

Figure 3

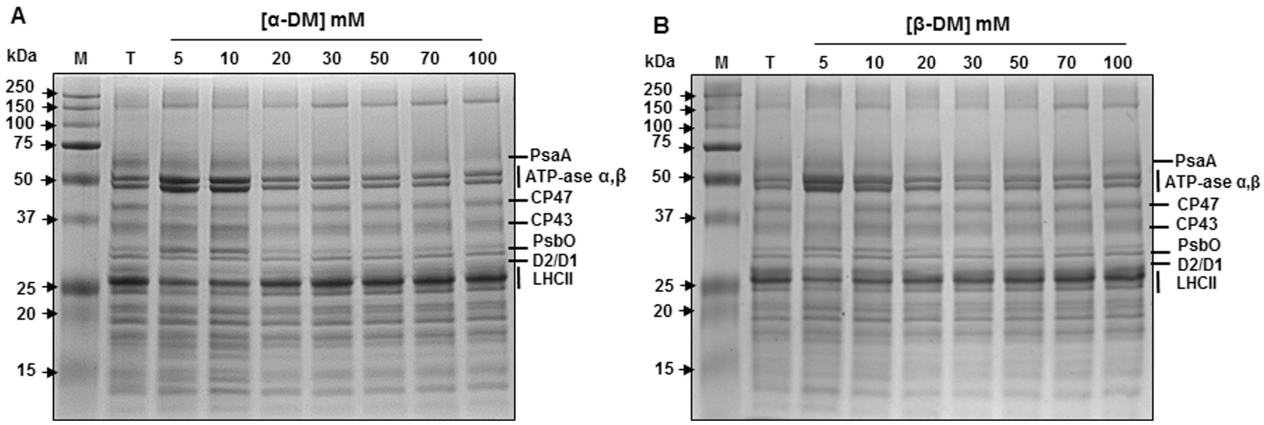

A
[α-DM] mM
kDa
M
T
5
10
20
30
50
70
100
250
150
100
75
50
37
25
20
15
PsaA
ATP-ase α,β
CP47
CP43
PsbO
D2/D1
LHCII
B
[β-DM] mM
kDa
M
T
5
10
20
30
50
70
100
250
150
100
75
50
37
25
20
15
PsaA
ATP-ase α,β
CP47
CP43
PsbO
D2/D1
LHCII

Figure 4

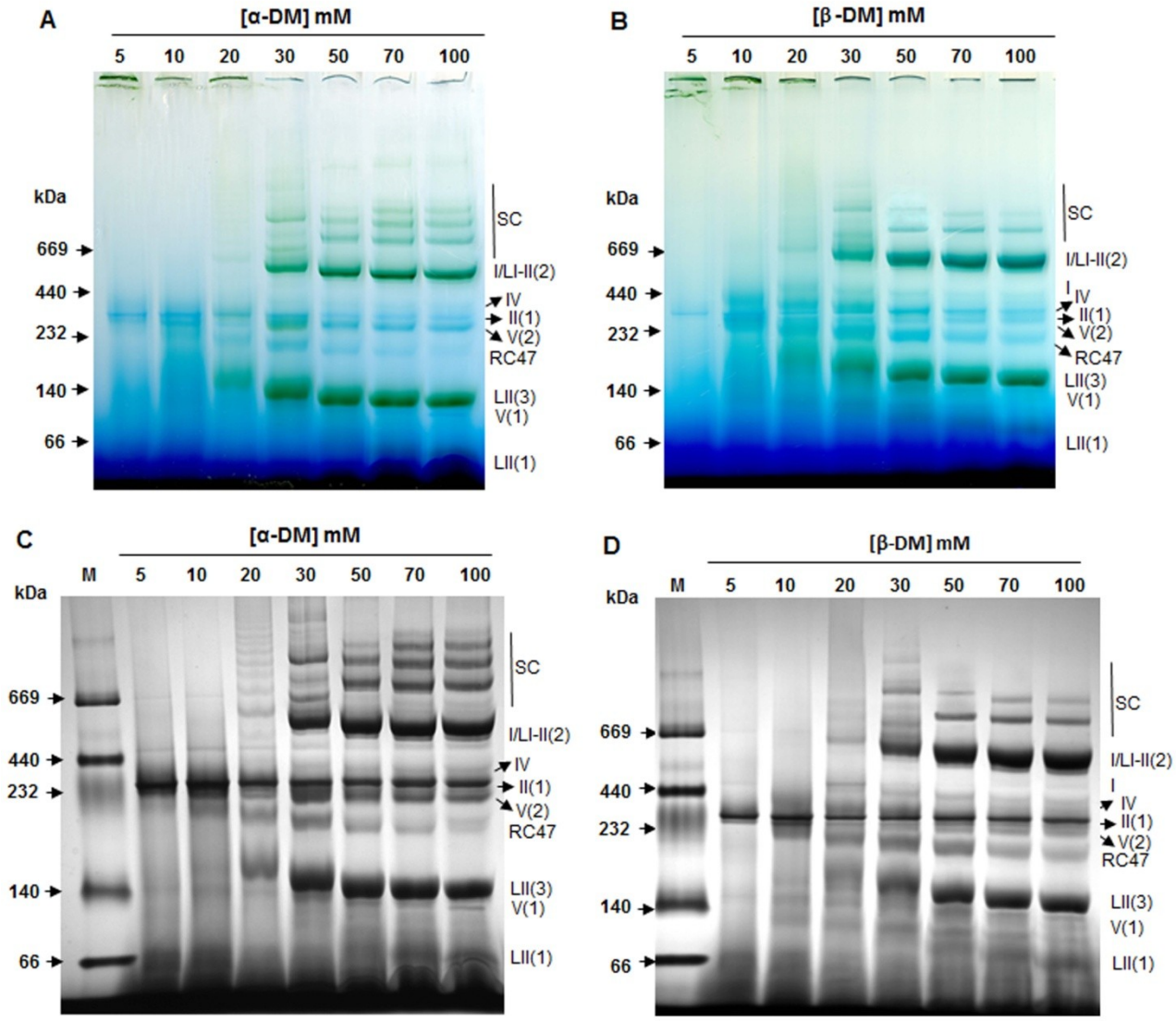

A
[α-DM] mM
5 10 20 30 50 70 100
kDa
669
440
232
140
66
SC
I/LI-II(2)
IV
II(1)
V(2)
RC47
LII(3)
V(1)
LII(1)
B
[β -DM] mM
5 10 20 30 50 70 100
kDa
669
440
232
140
66
SC
I/LI-II(2)
I
IV
II(1)
V(2)
RC47
LII(3)
V(1)
LII(1)
C
[α-DM] mM
M 5 10 20 30 50 70 100
kDa
669
440
232
140
66
SC
I/LI-II(2)
IV
II(1)
V(2)
RC47
LII(3)
V(1)
LII(1)
D
[β-DM] mM
M 5 10 20 30 50 70 100
kDa
669
440
232
140
66
SC
I/LI-II(2)
I
IV
II(1)
V(2)
RC47
LII(3)
V(1)
LII(1)

Figure 5

Figure 6

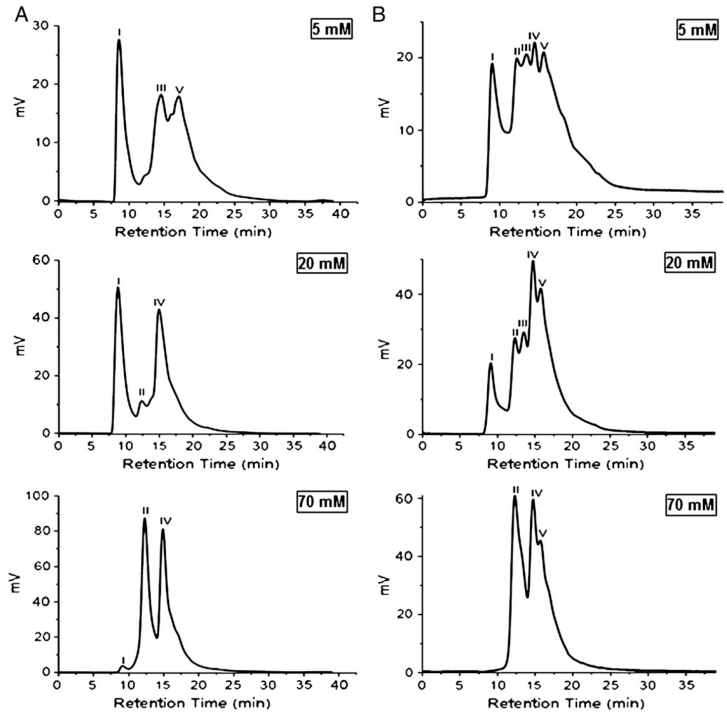
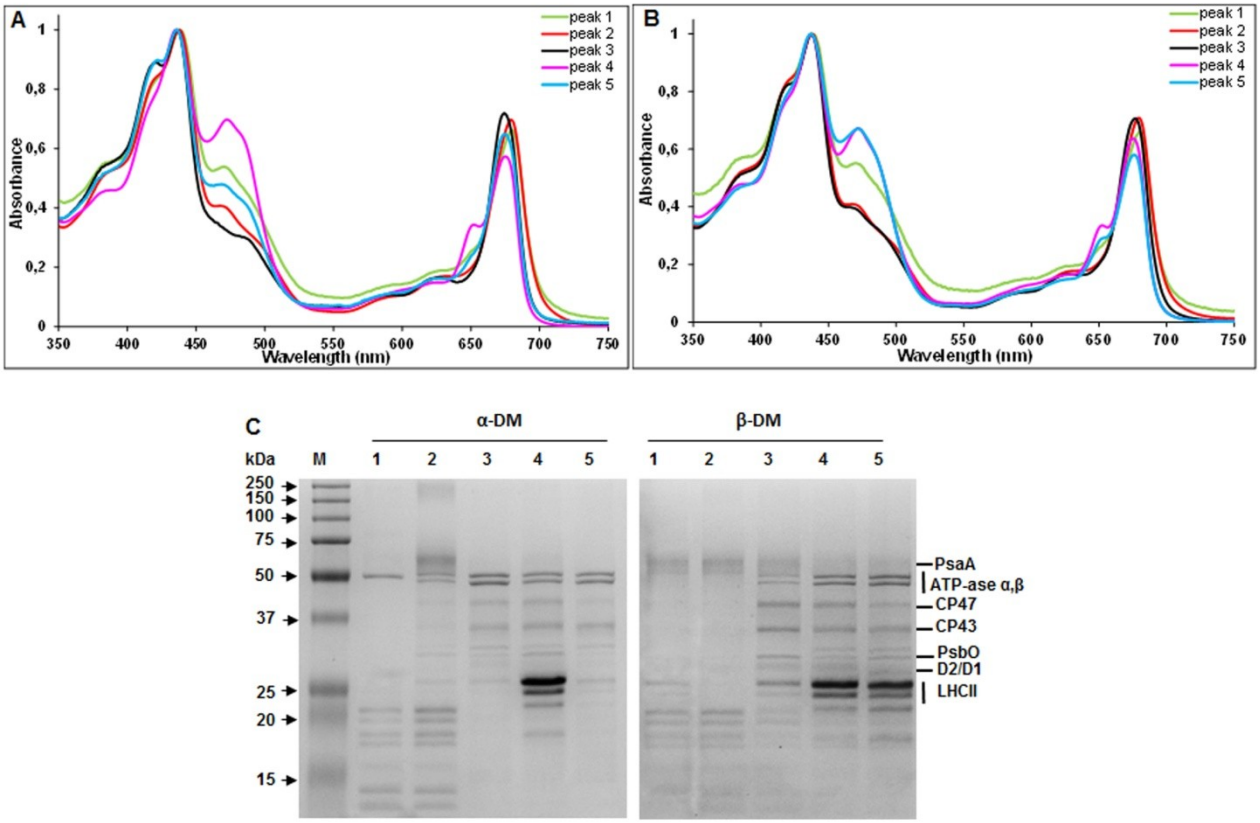


Figure 7


A
B
peak 1
peak 2
peak 3
peak 4
peak 5
Absorbance
Wavelength (nm)
C
α-DM
β-DM
kDa
M
1
2
3
4
5
250
150
100
75
50
37
25
20
15
PsaA
ATP-ase α,β
CP47
CP43
PsbO
D2/D1
LHCII