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Membrane protein isolation by in situ solubilization, partitioning and affinity adsorption in aqueous two-phase systems Purification of the human type 1 11β-hydroxysteroid dehydrogenase

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Abstract

Recently developed aqueous two-phase systems based on non-ionic detergents and polymers are suitable for the separation of membrane proteins. Moreover, within this relatively membrane protein "friendly" environment, changes in temperature can be controlled and stabilizing agents may be added to ensure integrity of the target protein during isolation. Here, we use aqueous two-phase partitioning for the isolation of membrane bound 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). Different detergents were used to find optimal conditions regarding solubilization and retaining target protein activity. We explored in situ solubilization by adding detergent directly to the aqueous two-phase system, as well as a batch metal affinity capture step of 6xHis tagged 11β-HSD1 in the two-phase system. The use of detergent/polymer two-phase systems resulted in a specific enzyme activity of 3840 nmol mg^{-1} min^{-1} of the target membrane protein compared to a conventional purification protocol where a specific enzyme activity of 1440 nmol mg^{-1} min^{-1} was achieved.

Keywords: Aqueous two-phase systems; Affinity adsorption; *Pichia pastoris*; Enzymes; Membrane proteins

1. Introduction

Up to 40% of all proteins in eukaryotic cells are integrated into biological membranes [1,2]. These membrane proteins are often multimeric complexes and peripherally attached to or more commonly inserted into the lipid bilayer of cell membranes and are involved in mediating different signals across the biomembrane [3]. Membrane proteins often act as drug targets and therefore belong to an interesting class of proteins to study, especially for pharmaceutical purposes. Due to their hydrophobicity, membrane proteins are difficult to handle outside the endogenous membrane and often require detergent for extraction and stabilisation [4,5]. New approaches to the isolation of membrane proteins offer access to pure protein for structural and functional membrane protein studies.

Aqueous two-phase systems were introduced as a separation method in the mid-1950s by Albertsson [6,7] and has since then been applied to a large number of different materials, such as plant and animal cells, microorganisms, viruses, chloroplasts, mitochondria, membrane vesicles, proteins and nucleic acids [8]. Aqueous two-phase systems are formed when for example two hydrophilic polymers such as poly(ethylene glycol) (PEG) and dextran are mixed over certain concentration ranges [8,9]. The systems contain about 80–95% water and at equilibrium, each phase is enriched in one of the polymers.

Non-ionic detergents with low critical solution temperature (cloud-point), e.g. Triton X-114, also form two-phase systems at specific temperatures [10]. Bordier showed earlier that above the cloud-point temperature a two-phase system is

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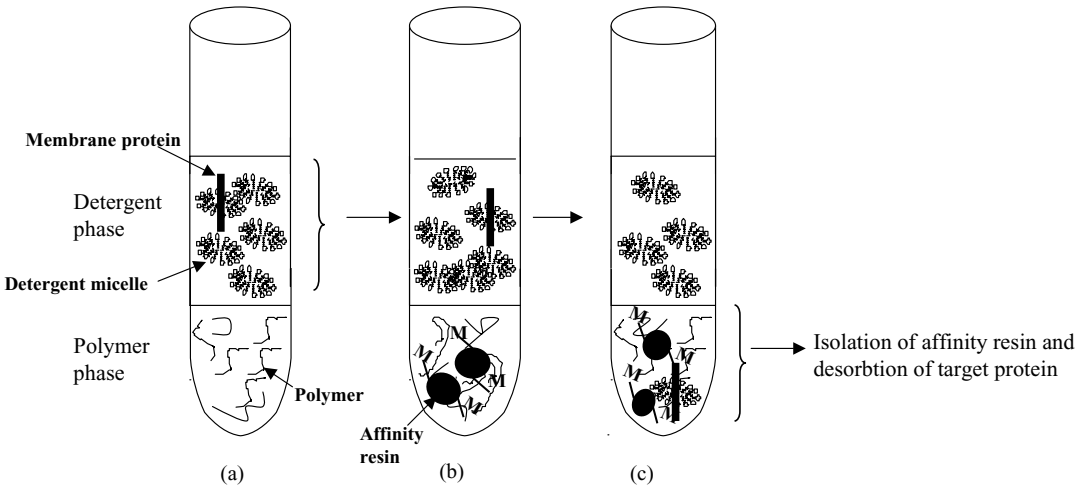

Fig. 1. Affinity partitioning of His-tagged membrane proteins in polymer/detergent aqueous two-phase system. Microsomes are partitioned directly into the two-phase system. (a) In a detergent/polymer aqueous two-phase system membrane proteins are partitioned into the detergent enriched phase. Micelles and membrane protein (■) are shown in the detergent phase. (b) To achieve a selective partitioning of the his-tagged target protein, the detergent phase with the target protein is added to a new polymer phase containing metal affinity resin (●-M). (c) After mixing and separation of system (shown in b) a two-phase system is formed and due to gravity the metal affinity resin will settle to the lower polymer enriched phase. Thus, a specific extraction of the target protein is achieved in the system. The affinity resin is separated from the two-phase system and washed before desorbtion of the target protein.

formed where one detergent-enriched phase is in equilibrium with a detergent-depleted phase. This system has been used for the separation of membrane proteins from hydrophilic proteins [11]. However, the cloud-point technique has some disadvantages. The selectivity between different membrane proteins is low since most of the membrane proteins are partitioned to the same phase. In addition, there are only a few non-ionic detergents that have the characteristic features that may form a two-phase system and in some cases, a high temperature must be used to achieve the formation of a two-phase system; this may result in an inactive protein.

Non-ionic detergents from for example the Triton series, alkylglucosides, alkyl(polyethylene oxide) (C_mEO_n) also form two-phase systems when mixed with polymers (e.g. dextran or PEG). When mixing these detergent and polymer solutions above certain concentrations, a detergent/polymer system is formed where one phase is enriched with the detergent and the other phase is enriched with the polymer. Thus, both phases contain detergent over the critical micelle concentration (CMC). By introducing this kind of system it is possible to increase the number of possible detergents to work with. In addition, it allows for decreasing the temperature at which the system is formed [12,13] which is favourable for keeping protein stability and activity. Different systems containing polymer/detergent/water have been characterized [12–15] and used for membrane protein purification.

In this report we present the isolation of an N-terminally 6xHis-tagged-11β hydroxysteroid dehydrogenase type 1 (N-His-11β-HSD1) that has been over expressed in *Pichia pastoris* yeast cells. Microsomal fractions of the over expressed enzyme were first added to an aqueous two-phase system for in situ solubilisation and phase separation and then combined with batch metal affinity adsorption (Fig. 1). The only earlier purification of full-length 11β-HSD1 was performed by metal affinity chromatography [16].

2. Experimental

2.1. Chemicals

Dextran T-500 was purchased from Amersham Biosciences (Uppsala, Sweden). C12EO5 and C12EO8 were from Anatrace (Maumee, OH, USA). Nonidet P-40 was from Calbiochem (San Diego, CA, USA). *n*-Dodecyl β-D-maltoside and Triton X-100 were purchased from Roche Diagnostics Scandinavia (Bromma, Sweden). Tween 20, Tween 80, Triton X-114 and C12EO30 were from Sigma–Aldrich Sweden (Stockholm, Sweden). Complete EDTA-free protease inhibitor was from Roche Diagnostics (Mannheim, Germany).

2.2. Strain and plasmid

Cloning of the human 11β-HSD1 and the generation of yeast clones has been described in detail previously by Nobel et al. [16]. In brief, the human 11β-HSD1 gene was cloned from HepG2 cells and fused to an N-terminal 6xHis tag containing a protease 3C cleavage site. This construct was inserted into the vector pPIC3.5K (Invitrogen, Groningen, The Netherlands) to obtain the recombinant plasmid pMB1250. This plasmid was linearized and transformed into the *P. pastoris* strain GS115 that was used as a host in the

cloning procedure according to the supplier's protocol (Invitrogen, Groningen, The Netherlands).

2.3. Protein expression

The recombinant *P. pastoris* strain GS115 expressing the N-terminal 6xHis tagged human 11β-HSD (pMB1250) was cultured in a fermentor at similar conditions as described [16]. Induction of protein expression was performed by additions of methanol starting at an A_{600} of 60–110. Cells were harvested 40 h after the initial addition of methanol by centrifugation and stored at −20 °C in equal parts of cell aliquots and freezing buffer (40 mM sodium phosphate, pH 7.0 and 30% glycerol).

A purified N-His-11β-HSD1, lacking the transmembrane domain (N-His-11β-HSD1 ΔTm) expressed in *Escherichia coli*, was used as a reference protein [17].

2.4. Preparation of microsomes

The frozen cell suspensions were diluted in 20 mM sodium phosphate, pH 7.5 at a ratio of 1:2.33. Complete EDTA-free protease inhibitor and 1 mM EDTA were subsequently added. Microsomes from this cell suspension were prepared by continuous sonication using a Vibra-Cell 600 W (Sonics & Materials, USA) chilled with cool water and operating at a flow rate of 15 ml h^{-1}. The sonication intensity was set to 100% output. The cell homogenate was centrifuged at 9000 g for 15 min and the pellet was discarded. The supernatant, containing the microsomes, was further ultracentrifuged at 140 000 g for 60 min in order to concentrate the microsomes. The obtained pellet was dissolved in preparation buffer (40 mM sodium phosphate, pH 7.5, 5% glycerol and 1mM EDTA), divided in aliquots and stored at −70 °C until further use.

2.5. Protein determination

The protein content of all samples was determined by BCA protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as protein standard.

2.6. Protein solubilization prior two-phase separation

Microsome preparations were thawed and centrifuged at 128 000 × *g* for 60 min. The subsequent microsome pellets were then dissolved to a final protein concentration of 2.5–3.6 mg ml^{-1} in 20 mM sodium phosphate, pH 7.5, 500 mM sodium chloride and 5% glycerol, including complete EDTA-free protease inhibitor. For the solubilization screen, detergent was added to a final concentration of 1% (w/v). The different detergents screened for are listed in Table 1. The samples were mixed and subsequently incubated for 60 min at 4 °C by inversion. The unsolubilized material was removed by ultracentrifugation at 128 000 g for 60 min. The supernatant was subjected to analysis and partitioned in an appropriate two-phase system.

2.7. In situ protein solubilization in aqueous two-phase system

The two-phase system containing detergent/polymer/water for in situ solubilization was prepared with a total weight of 3 g, containing 8% Tween 20 and 6% Dextran T-500 based on phase diagrams performed by Sivars and Tjerneld [12]. The microsome pellet was dissolved in 20 mM sodium phosphate, pH 7.5, 500 mM sodium chloride and 5% glycerol before the addition of Dextran T-500 and Tween 20. The system contained approximately 11 mg microsomes/3 g system. The two-phase system was briefly vortexed and incubated for 60 min at 4 °C by gentle inversion.

2.8. Two-phase partitioning and batch affinity adsorption

Phase separation of the two-phase system was achieved by centrifugation at 1600 × *g* for 10 min. In addition to the two liquid phases a pellet was formed containing unsolubilized material. From the separated phases the detergent top phase was collected and added to a new pre-chilled bottom phase including additional 0.5 ml BD Talon cobalt ion-based resin (Clontech, Palo Alto, CA, USA). Prior to addition to the

Table 1
Solubilzation efficiency studies of N-His-11β-HSD1 from microsomes of *P. pastoris* using nine different detergents at 1% (w/v) detergent concentration

Detergent	Molecular mass (g mol^{-1})	CMC[a] (mM)	Detergent concentration[b] (mM)	Protein concentration (μg ml^{-1})
Nonidet P-40	607	0.30	16.5	675
Dodecyl maltoside	510	0.18	19.6	1650
Tween 80	1310	0.012	7.6	430
Tween 20	1228	0.06	8.1	850
Triton X-114	514	0.21	19.5	800
Triton X-100	674	0.20	14.8	850
C12EO30	1200	0.09	8.3	1350
C12EO8	539	0.09	18.7	1580
C12EO5	407	0.65	24.6	1610

Western blot analysis is shown in Fig. 3.

[a] CMC: critical micelle concentration.

[b] Detergent concentration in mM at 1% (w/v) detergent concentration.

new bottom phase, the metal affinity resin was washed with 20 mM sodium phosphate, pH 7.5, 500 mM sodium chloride 5% glycerol and 5 mM imidazole, by 20 min inversion, followed by centrifugation at 700 g for 5 min at 4 °C. The new two-phase system was briefly vortexed and incubated for 20 min at 4 °C by gentle inversion. Separation was achieved by centrifugation at 1600 × *g* for 10 min. The top phase was discarded. The bottom phase containing affinity resin was collected. The resin was first washed with 20 mM sodium phosphate, pH 7.5, 0.02% Tween 20 and 5 mM imidazole and spun at 1400 × *g* for 5 min; then, capture of the protein from the bottom phase was achieved by batch mode desorbtion from the Talon resin. A 20 min incubation of the resin was performed in 20 mM sodium phosphate, pH 7.5, 0.02% Tween 20 and 100 mM imidazole to remove unspecifically bound proteins. The target protein was then eluted in 20 mM sodium phosphate, pH 7.5, 0.02% Tween 20 and 250 mM imidazole. The last step was repeated once. The protein sample was aliquoted and stored at −20 °C until further analysed.

2.9. Assay enzymatic activity

Activity kinetics of 11β-HSD1 was measured according to Hult et al. [18], with minor modifications. Eluted fractions were incubated in reaction mixture (50 mM Tris–HCl, pH 8.0 and 1 mM EDTA) including 100 μM cortisol (hydrocortisone) and 200 μM of the co-factor, $NADP^+$. The enzymatic dehydrogenase reaction is the conversion of cortisol to cortisone. The reaction was initiated by the addition of 20 μl protein extract, containing approximately 1–20 μg microsomal protein (different protein concentrations in the samples were accounted for in the calculation of the kinetics). The reaction was terminated after 40 min of incubation at 37 °C by adding 3 volumes of excess ice-cold acetonitrile, followed by centrifugation for 10 min at 1300 × *g*. The extracted supernatant was analysed by C_{18}-RP-HPLC where substrate and product were separated in 30% acetonitrile and 0.1% ammonium acetate, pH 7.0 as previously been described [18].

2.10. Protein analysis

Samples with high detergent content, i.e. from the detergent screen, were desorbed of access detergent by the use of hydrophobic beads (Pierce, Rockford, IL, USA) prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Gel electrophoresis was carried out on 4–12% Bis-Tris SDS-NuPAGE (Invitrogen, Carlsbad, CA, USA) and proteins were transferred by semi-dry blotting [19] and probed with anti-His primary antibodies 1:200 (Dianova, Hamburg, Germany) followed by anti-mouse horseradish peroxidase (HRP) secondary antibodies 1:2000 (Amersham, Buckingham, UK). Enhanced chemoluminescence ECL (Amersham, Buckingham, UK) was used for the detection.

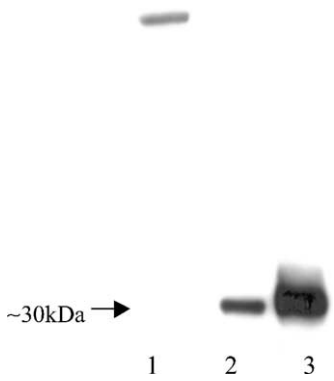

Fig. 2. Western blot analysis on starting material for N-His-11β-HSD1 isolation. Lanes: 1 = cellulose binding model lipase from *P. pastoris* cells not expressing N-His-11β-HSD1; 2 = N-His-11β-HSD1 from *E. coli*; 3 = N-His-11β-HSD1 solubilised from *P. pastoris* microsomes in 1% Tween 20.

3. Results

The *P. pastoris* strain G115 was chosen as an expression system based on previous successful expression of human 11β-HSD1 in this system [16,18]. The microsomal fractions prepared from *P. pastoris* cells overexpressing recombinant N-terminally 6xHis-tagged 11β-HSD1 (N-His-11β-HSD1), contained high amounts of enzymatically active enzyme confirming the quality of the starting material. Fig. 2 illustrates the overexpression of N-His-11β-HSD1 as visualized by Western blot analysis. To characterize the migration pattern of the protein overexpressed in the yeast cells, a purified fraction of N-His-11β-HSD1 ΔTm overexpressed in *E. coli* cells was run in parallel (Fig. 2). Previous data show that the protein expressed in *P. pastoris* is not glycosylated [20].

A screening for most effective detergent for solubilization was performed with 9 different detergents (Table 1). Protein analysis of each sample showed that the total amount of solubilized protein differed between the detergents. All detergents were effective in solubilizing proteins from the microsomal membranes. The detergents dodecyl maltoside, C12EO5, C12EO8, and C12EO30 were most effective in solubilizing total amount of proteins from the membranes (Table 1). However, this did not entirely correlate with the solubilization of N-His-11β-HSD1 since Tween 20 and Triton X-100 also provided substantial solubilization of the target protein as determined by Western blot analysis (Fig. 3).

Fig. 3. Western blot analysis of 9 different detergents for the optimal solubilization of N-His-11β-HSD (see Table 1). Lanes: 1 = Nonidet P-40; 2 = Dodecyl maltoside; 3 = Tween 80; 4 = Tween 20; 5 = Triton X-114; 6 = Triton X-100; 7 = C12EO30; 8 = C12EO8; 9 = C12EO5.

Table 2
Enzyme activity of N-His-11β-HSD1 after solubilization in five different detergents at 1% (w/v) detergent concentration

Detergent	Protein concentration[a] ($\mu g\,ml^{-1}$)	Total activity[a] ($nmol\,min^{-1}$)	Specific activity[a] ($nmol\,mg^{-1}\,min^{-1}$)
Dodecyl maltoside	1700	2.4	1.4
Tween 20	950	1.7	1.7
Triton X-100	1600	2.3	1.4
C12EO8	1220	3.0	2.5
C12EO5	650	0.6	0.7

[a] Protein concentration and enzyme activity in the supernatant after solubilization and centrifugation.

Moreover, the degree of solubilization of N-His-11β-HSD1 obtained with Tween 20 was clearly higher than with the other detergents used as seen in the Western blot analysis.

The preservation of enzymatic activity of solubilized N-His-11β-HSD1 was studied with five detergents that were effective for solubilization: Triton X-100, Tween 20, dodecyl maltoside, C12EO8 and C12EO5. The enzyme activity, determined by substrate conversion of cortisol to cortisone, is shown in Table 2. The total activity was highest in the samples solubilized with C12EO8 followed by dodecyl maltoside, Triton X-100 and Tween 20. Calculated on a specific activity basis of the samples, enzyme activity was again highest in the samples solubilized with C12EO8 followed by Tween 20 (Table 2).

Five detergents were tested for developing a suitable detergent/polymer aqueous two-phase system: Triton X-100, Tween 20, dodecyl maltoside, C12EO8, and C12EO5. As water soluble polymer Dextran 500 was used. Earlier determined phase diagrams for two-phase systems with Dextran 500 and detergents Triton X-100, Tween 20, C12EO8 and C12EO5 were used [12]. In the dodecyl maltoside/Dextran 500 system, too high detergent concentration was needed to obtain phase separation due to that both detergent and polymer were sugar based [21]. Solubilization of microsomes was performed in situ in the two-phase systems. In the different detergent/Dextran 500 systems target protein was partitioned to the detergent phase and the enzyme activity was preserved. Due to low cloud point C12EO5 solutions might phase separate at temperatures that make this detergent difficult to handle. In the preparation of the aqueous two-phase systems Tween 20 was the least viscous and thus easiest to work with. Based on this, as well as on the high recovery of enzyme activity (Table 2), Tween 20 was selected as detergent for solubilization and phase separation.

In the final isolation of N-His-11β-HSD1 by detergent/polymer two-phase system partitioning combined with batch affinity adsorption (Fig. 1) the Tween 20/Dextran 500 system was used. After in situ solubilization and phase separation, much of the total protein was partitioned to the bottom Dextran phase but the majority of N-His-11β-HSD1 (Fig. 4) as well as activity (>75%) was found in the top Tween 20 phase. New bottom phase with cobalt based resin was added to the detergent phase containing target protein. After mixing and phase separation, the affinity resin was collected in the bottom Dextran phase. For elution of target protein the resin was applied on a column and the specific enzyme activity was calculated to 1470 and 2370 $nmol\,mg^{-1}\,min^{-1}$ in the first and second eluate, respectively (total 3840 $nmol\,mg^{-1}\,min^{-1}$). The method described in Fig. 1 was compared to an alternative purification method for membrane proteins, comprising solubilization, centrifugation and affinity adsorbtion. A sample solubilized in 1% Tween 20 as described above, had an enzymatic activity similar to the top phase of the Tween 20/Dextran 500 two-phase system but contained much more contaminating proteins. After subjection of this sample to cobalt based resin, the specific activity was 1320 and 120 $nmol\,mg^{-1}\,min^{-1}$ in the first and second eluate, respectively (total 1440 $nmol\,mg^{-1}\,min^{-1}$). Thus, the use of a detergent/polymer two-phase system resulted in higher recovery of enzyme activity compared to a separation protocol where the metal affinity resin was added directly in a detergent solubilized membrane preparation.

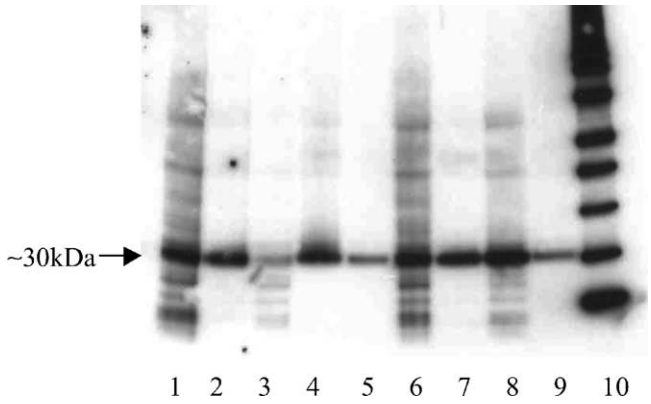

Fig. 4. N-His-11β-HSD1 isolation by aqueous two-phase separation. Lanes 1–5 are from the two-phase separations combined with batch affinity adsorption and lanes 6–10 from a protocol where solubilisation was directly followed by batch affinity adsorption. Lanes 1 = start material; 2 = top Tween 20 phase; 3 = bottom Dextran 500 phase; 4 = first eluate after adsorption; 5 = second eluate after adsorption; 6 = start material; 7 = first eluate after adsorption; 8 = N-His-11β-HSD1 in 1% Tween 20 (control sample after solubilisation); 9 = second eluate after adsorption.

4. Discussion

The presented purification procedure is fast, simple, and preserves protein stability. This is possible due to the use

of optimal detergent conditions followed by two-phase partition. This reduces both time and the number of steps needed, both leading to pure more active protein. Structural intactness is essential for activity of a membrane protein, hence the importance of an initial screen. Screening for optimal detergent conditions was evaluated with Western blotting and according to preserved enzyme activity.

Narrowing the range of possible detergents to five or less allows for ease of adaptation to two-phase partition [12]. Initially the detergents dodecyl maltoside and C12EO8 seemed most promising, however better overall compatibility was shown by the Tween 20 system. Dodecyl maltoside solubilized more non-target membrane proteins, hence the high total protein content. Full-scale isolation studies with detergent/polymer aqueous two-phase systems were performed with Tween 20 that preserved activity. In the final step for the purification we included batch affinity adsorption utilizing metal affinity of the target protein. This saved time without sacrificing yield compared to conventional immobilized metal affinity chromatography.

There are several advantages of working with detergent/polymer based aqueous two-phase systems. In contrast to cloud-point systems [11] there is no need to increase the temperature to obtain a two-phase system. Phase separation is due to incompatibility between detergent micelles and polymer, i.e. basically the same mechanism as for polymer/polymer aqueous two-phase system (e.g. PEG/Dextran systems [12,21]. Partitioning can thus be carried out at cold room temperatures and the systems are easy to handle at low temperatures. Since detergent is present in the aqueous two-phase system a solubilization of membrane proteins can be made directly by adding a membrane preparation into the two-phase system. Membrane proteins are enriched by partitioning to the detergent phase of the system while water-soluble proteins are partitioned to the more hydrophilic polymer phase [12,13].

The protein N-His-11β-HSD1 could be efficiently solubilised with the detergent Tween 20. Also, in presence of Tween 20 the activity of the target protein was well preserved. These findings made Tween 20 a suitable detergent in an aqueous two-phase system together with Dextran 500 as polymer. After adding microsomes into the detergent/polymer system the majority of N-His-11β-HSD1 was partitioned into the top detergent phase. To obtain a specific purification of the His-tagged target protein cobalt based resin was added directly into the system, i.e. the resin was included in a new polymer phase that was equilibrated with the detergent phase (see Fig. 1). The His-tagged protein interacted with the resin and a specific partitioning of the target protein was achieved due to gravity settling of the resin to the lower polymer phase. By combining a detergent/polymer aqueous two-phase system together with cobalt based resin a selective partitioning of a specific target protein could be obtained in a very rapid and efficient way.

In conclusion, the principle shown here demonstrates a combination of two separation steps in one, i.e. phase partitioning combined with metal affinity adsorption. For purification of N-His-11β-HSD1 this method resulted in higher recovery of preserved enzyme activity. Detergent/polymer aqueous two-phase systems offer a basic methodology for partitioning and isolation of membrane proteins in a very efficient way. Developing a new partition-based protocol does not require more time or cost than for classical protocols. Screening for optimal detergent in order to obtain maximal solubilization and preserving enzyme activity are recommended prior application to a two-phase system. Also, along the time when new detergents and polymers are being discovered these can be used to create and design new detergent/polymer systems. A very attractive feature of the aqueous phase partitioning approach for membrane protein isolation is that combination of the liquid based processes in one vessel makes it even more rapid, simple and flexible.

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