Solubilization of the ecdysone binding protein from anterior silk gland cell membranes of the silkworm, bombyx mori

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Solubilization of the Ecdysone Binding Protein from Anterior Silk Gland Cell Membranes of the Silkworm, *Bombyx mori*

Mohamed Elmogy†, Masafumi Iwami and Sho Sakurai*

Division of Life Sciences, Graduate School of Science and Technology, Kanazawa University, Kakumamachi 920-1192, Japan

We previously provided preliminary evidence for the presence of a putative membrane ecdysone receptor (mEcR) anchored in the plasma membranes of anterior silk glands (ASGs) in *Bombyx mori*. This receptor may act in concert with the conventional EcR in 20E-dependent programmed cell death of these glands. We report here, for the first time, the solubilization of mEcR from ASG membranes using the zwitterionic detergent CHAPS in the presence of NaCl. Our results show by ligand binding assay that mEcR solubilized this way is functionally active and retains 75% of its native binding activity. We also defined experimental conditions that yielded protein/detergent complexes with partial binding activity, which makes it possible to purify the membrane-bound ecdysone binding protein.

Key words: anterior silk gland, ecdysteorid, membrane ecdysone receptor, detergent, solubilization, *Bombyx mori*

INTRODUCTION

Recognition of the complexity of steroid signaling has substantially increased in recent years. Steroids enter cells and bind to ligand-regulated transcription factors (Beato and Klug, 2000; Henrich, 1999). However, steroids also exert non-genomic effects that occur independently of gene transcription (Falkenstein *et al*., 2000). Recent studies suggest that steroid hormones bind receptors on cellular membranes to gain access to the intracellular compartment and modulate cellular functions (Watson and Gametchu, 1999). Typically, these effects are initiated at the plasma membrane and result in the regulation of membrane protein-mediated intracellular signaling pathways (Sarramegna *et al.,* 2006). The dependence of the native conformation of these proteins in the hydrophobic intra-membrane environment has complicated attempts to isolate those integral surface receptor molecules (Neugebauer, 1990).

The anterior silk glands (ASGs) of the silkworm, *Bombyx mori*, begin to undergo programmed cell death (PCD) in response to a high hemolymph concentration of ecdysteroid, which induces pupal metamorphosis (Terashima *et al.,* 2000). The biologically active form of ecdysone, 20-hydroxyecdysone (20E), binds to a functional nuclear ecdysone receptor consisting of an ecdysone receptor (EcR) and its heterodimeric partner, Ultraspiracle (USP), and thereby controls the transcriptional activity of target genes (Riddiford *et al.,* 2000). We previously provided preliminary evidence for the presence of a putative membrane ecdysone receptor (mEcR) located in the plasma membranes of *B. mori* ASGs, which may act in concert with the conventional nuclear EcR (Elmogy *et al.,* 2004, 2006). Thus, to further characterize mEcR, we tried to solubilize from ASGs a functional mEcR retaining its binding activity to ecdysteroids. Since no single detergent or solubilization scheme is universally applicable to all membrane proteins (Tanford and Reynolds, 1976; Kerkhoff *et al*., 1996, 2000), the important aspect of the present approach is the use of an apporopriate detergent as a solubilizing agent.

Detergent solubilization is a crucial step for the purification and characterization of membrane-bound receptors. This is effectively accomplished by amphiphilic detergents in a process known as solubilization (Banerjee, 1999; Hjelmeland and Chrambach, 1984; Jones *et al*., 1987). Solubilization of a membrane protein that retains its biological activity is a formidable challenge, since many detergents irreversibly denature membrane proteins (Garavito and Ferguson-Miller, 2001).

The mild, nondenaturing zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) is one of the most commonly used detergents in membrane biochemistry (Hjelmeland, 1980). CHAPS is a derivative of naturally occurring bile salts and has useful features of both the bile salt hydrophobic group and the *N*-alkyl sulfobetaine-type polar group (Hjelmeland, 1980; Hjelmeland and Chrambach, 1984). Also, CHAPS has very low absorbance at 280 nm (unlike TritonX-100) and does not have circular dichroic activity in the far UV region, making it ideal for optic studies of proteins. These factors have led to extensive use of this detergent in solubilization of

* Corresponding author. Phone: +81-76-264-6250;
Fax : +81-76-264-6255;
E-mail : ssakurai@kenroku.kanazawa-u.ac.jp

† Present address: Department of Entomology, Faculty of Science, Cairo University, Giza, Egypt
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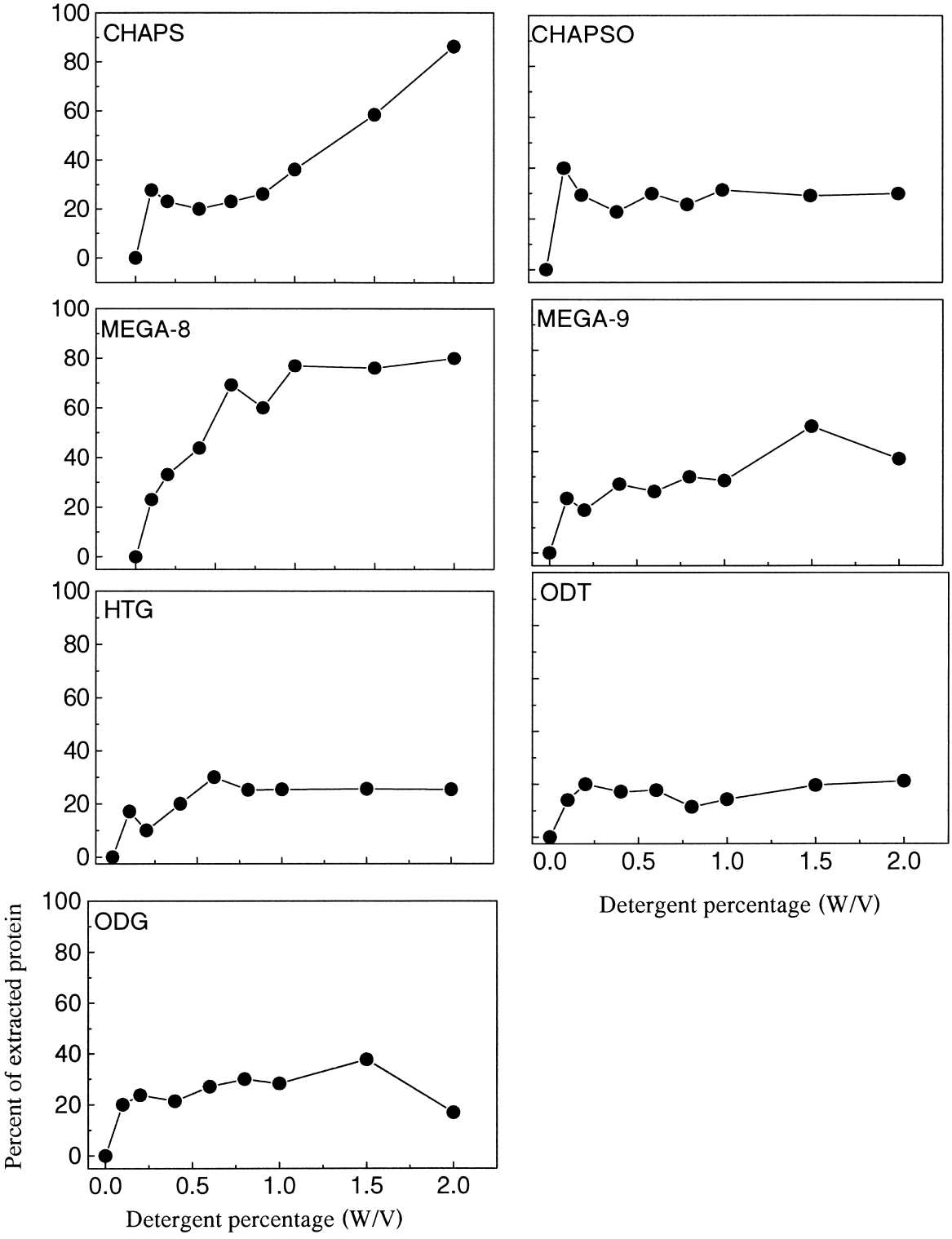
membrane receptors (Banerjee *et al.,* 1995; Ofri *et al.,* 1992).

Here, we report the solubilization of mEcR from *B. mori* ASGs using CHAPS in the presence of NaCl, followed by dissociation of protein/lipid/CHAPS mixed micelles into protein/CHAPS complexes, which appeared in high enough quantity to be adopted for protein purification. We also show by ligand-binding assay that mEcR solubilized by this way is functionally active.

(A)



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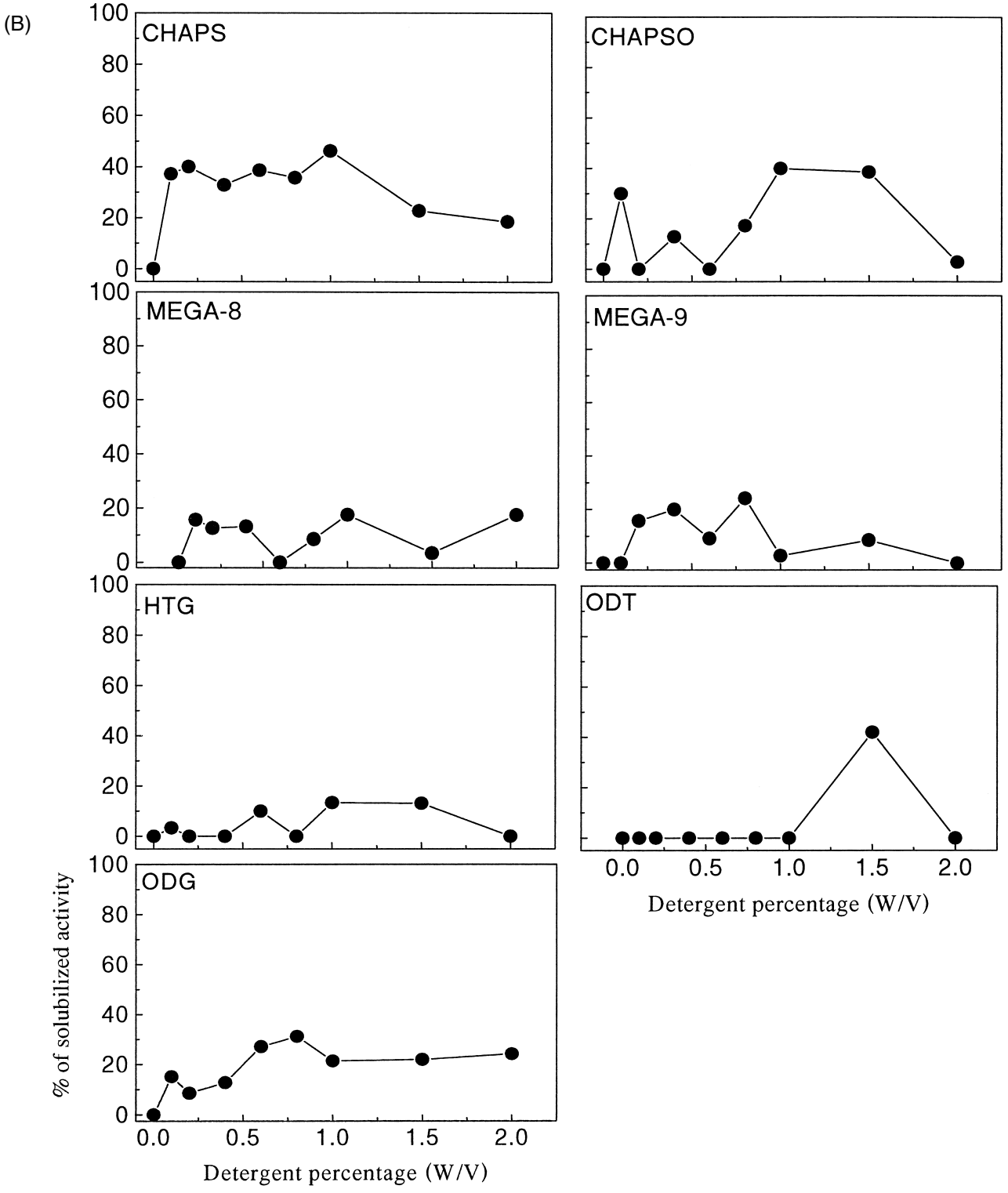

Fig. 1. Initial solubilization using zwitterionic and nonionic detergents. Membrane fractions from ASGs, prepared as described in Materials and Methods, were suspended in assay buffer to a final protein concentration of 1 mg/ml and treated with varied concentrations of the detergents CHAPS, CHAPSO, MEGA-8, MEGA-9, HTG, OTG, and OG. **(A)** Extracted proteins in supernatants (ordinate) are shown as a percentage of those in the crude membrane preparations. **(B)** Solubilized activity (ordinate) is shown as the percentage of binding activity in the supernatant relative to that in the crude membrane preparation. Each datum point is the mean of duplicate determinations.

MATERIALS AND METHODS

Animals and ASGs

Larvae of *B. mori* (Kinshu x Showa, an F1 hybrid stock of Chinese and Japanese strains) were reared on an artificial diet (Silkmate, Nihon-Nosan-Kogyo, Yokohama, Japan) at 25°C under a 12-h light/12-h dark cycle (Sakurai, 1994). ASGs were dissected on the day of gut purge (Terashima *et al.,* 2000) and cultured separately in 0.3 ml Grace's insect culture medium (Gibco BRL, Rockville, MD) at 25°C for 18 h with 1 μM 20E, followed by culture in a hormone-free medium for further 12 h. Because the binding activity in membrane fractions prepared from cultured ASGs is higher than that in freshly dissected ASGs (Elmogy *et al.,* 2004), we mainly used cultured ASGs.

Hormones and reagents

The reagents 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxypropanesulfonic acid (CHAPSO), n-heptyl-β-D-thioglucoside (HTG), n-octanoyl-*N*-methylglucamide (MEGA-8), nonanoyl-*N*-methylglucamide (MEGA-9), n-octyl-β-D-glucoside, and n-octyl-β-D-thioglucoside were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ponasterone A (PonA, 25-deoxy-20-hydroxyecdysone) and 20E were obtained from Sigma (St.Louis, MO). Ecdysteroids were dissolved in ethanol and stored at –20°C until use. [^{3}H]PonA (200 Ci/mmol) was obtained from Perkin-Elmer Life Science (Boston, MA).

Preparation of crude membrane fraction

Cultured ASGs were washed three times with insect Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$). All subsequent procedures were performed at 4°C. ASGs were homogenized in seven volumes of assay buffer (20 mM Tris/HCl, pH 7.0, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 3 μg/ml pepstatin A, 3 μg/ml leupeptin) using a motor-driven, loose-fitting glass-plastic homogenizer at 1,000 rpm for 1 min. After centrifugation at 1,000×g for 10 min, the resulting pellet was suspended in the buffer and centrifuged at 1,500×g for 10 min. The pellet was suspended again in buffer and centrifuged at 1,800×g for 10 min. The resulting pellet was resuspended in buffer, homogenized again using an HG30 homogenizer (Hitachi) on ice, and centrifuged at 1,000×g for 10 min. The supernatant was centrifuged at 8,000×g for 10 min, and the resulting supernatant was centrifuged at 105,000×g for 5 h. The pellet was suspended in buffer, frozen with liquid nitrogen, and stored at –80°C until use. Crude membrane fractions of ASGs (1 mg/ml) were treated with different concentrations of individual detergents for 60 min at 4°C with constant stirring, then centrifuged at 105,000×g for 60 min at 4°C. The binding activity of the supernatant, measured immediately after centrifugation, was regarded as 'solubilized activity', and the protein amounts in the supernatant were regarded as extracted proteins. Protein amounts were measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA) with BSA as a standard.

Preparation of P1 and P2 membranes from crude membranes of ASGs

Crude membrane suspensions containing 5 mg/ml proteins were treated with solutions of high ionic strength (binding assay buffer containing 1M NaCl) for 60 min at 4°C. After centrifugation at 105,000×g for 1h, the resulting pellet (P1) was resuspended in the binding assay buffer to a final protein concentration of 20 mg/ml and stored in aliquots at –80°C. P1 membrane suspensions containing 5 mg/ml protein were each treated with 0.05% MEGA-8 at 4°C for 60 min with constant stirring and again centrifuged at 105,000×g for 60 min at 4°C. The resulting pellet (P2) was resuspended into binding assay buffer to a final protein concentration of 20 mg/ml and stored in aliquots at –80°C.

Solubilization of the membrane proteins

P1 and P2 membranes were resuspended in the binding assay buffer to a final protein concentration of 1 mg/ml and treated with combinations of increasing concentrations of CHAPS and NaCl. The mixtures were then incubated at 4°C for 60 min with constant stirring and centrifuged at 105,000×g for 60 min at 4°C. The binding activity in the supernatant, measured immediately after centrifugation, was regarded as 'solubilized activity'.

Binding assay

Specific binding of [^{3}H]PonA was assayed in the solubilization experiments by the use of modified dextran-coated active charcoal (DCC) (Yoshikuni *et al.,* 1993). The dialysed supernatants of the solubilized membrane fractions were incubated for 10 min at 25°C with 25 nM [^{3}H]PonA without or with a 1,000-fold molar excess of inert PonA. DCC buffer (200 μl; charcol-dextran in 20 mM Tris/HCl pH 7.0, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 3 μg/ml pepstatin A, 3 μg/ml leupeptin) was added, mixed, and incubated for 10 min at 4°C. After incubation, the mixture was centrifuged at 6,000×g for 10 min at 4°C. The radioactivity in the supernatants was then measured using a Beckman LS-700 counter with a dual-label program (Beckman-Coulter). The potency of DCC buffer for separating free [^{3}H]PonA was examined using different concentrations of DCC buffer at five combinations of charcoal / dextran concentrations (0.25% / 0.0025%, 0.5% / 0.0025, 1% / 0.025% , 0.5% / 0.05%, and 1% / 0.1%). The most potent concentration ratio of the DCC buffer was 1% charcoal: 0.025% dextran (data not shown), and this ratio was used for measurements of specific binding of [^{3}H]PonA.

RESULTS AND DISCUSSION

The purification of a membrane-bound protein requires the solubilization of proteins. Effective solubilization requires both the selection of a good detergent and the determination of appropriate solubilization conditions. We first examined seven detergents to extract proteins from membranes of freshly dissected ASGs (Fig. 1).

Among the detergents tested, CHAPS and MEGA-8 were the most effective for extracting membrane proteins from ASG membranes. Approximately 80% of proteins in the membrane suspension were extracted with 2% of CHAPS and 1% MEGA-8. Other detergents were not capable of effectively solubilizing proteins, as the maximum amount of extracted protein was less than 40% of proteins in the membrane suspension before the extraction (Fig. 1A). [^{3}H]PonA binding activities of the solubilized crude membrane fractions are shown as a function of increasing concentrations of various detergents in Fig. 1B. The specific activity solubilized was estimated by measuring the binding activity in the supernatants immediately after centrifugation of the mixture of membrane fractions with each detergent. The maximal solubilized binding activity was observed with 1% CHAPS and accounted for 46.2% of the binding activity in the crude membrane fractions (Table 1). However, the protein concentration in the supernatant with 1% of CHAPS was 36.2% of that in the crude membrane fractions. The maximal protein concentration solubilized in the supernatants was observed with 1% MEGA-8 and accounted for 76.9% of that in the crude membrane fractions. However, the solubilized binding activity at 1% of MEGA-8 was only 17.5% of the binding activity in the crude membrane fractions. This result indicates that a very low concentration, 0.05%, of MEGA-8 was useful for the initial dispersal of lipid and fragmentation of the membranes. Of the seven detergents, CHAPS,

Table 1. Concentrations of zwitterionic and nonionic detergents that show maximum percentage of solubilization.

Detergent	Detergent (w/v) for max. solubilization	% of solubilized binding activity*	% of solubilized proteins**
CHAPS	1	46.2	36.2
CHAPSO	1	40	31.4
n-octyl-bD-glucoside	0.75	31.2	30
MEGA-8	1	17.5	76.9
MEGA-9	0.75	24.2	30
n-cotyl-b-D-thioglucoside	1.5	42	19.6
n-heptyl-b-D-thioglucoside	1	13.3	25.4

* Percentage of specific binding activity of the solubilized proteins in the supernatants of that in the crude membrane fractions.

** Percentage of extracted protein concentrations in the supernatants of that in the crude membrane fractions.

CHAPSO, and OTG showed high percentages of specific binding, while CHAPS and CHAPSO solubilized moderate amounts of proteins (Table 1). Accordingly, we selected CHAPS for the initial solubilization of the ecdysone binding sites in membrane.

Solubilization of proteins in P1 and P2 membrane fractions

When crude membranes were extracted with 1M NaCl, most ecdysone binding activity occurred in the precipitate (P1). Since 1M NaCl is capable of separating peripheral proteins from the membrane, the P1 fraction may have consisted of integral membrane proteins, indicating that the ecdysone binding sites are associated with integral membrane proteins. After treatment of the P1 fraction with 0.05% MEGA-8, the major binding activity was detected in the precipitate (P2), showing that 0.05% MEGA-8 did not solubilize integral membrane proteins but merely fragmented membranes (Elmogy *et al*., 2004). Based on these preliminary results, we tried to find the optimal conditions for solubilization of the ecdysone binding sites using P2 pellets prepared according to our previous results (Elmogy *et al.,* 2004). P1 pellets were resuspended in the assay buffer and treated with combinations of increasing CHAPS and NaCl concentrations for 60 min at 4°C. After centrifugation of the suspension, the binding activities in the resulting supernatants were assayed (Fig. 2). Solubilization of PonA binding activity depended on the ionic strength of the solubilization buffer, with the maximum binding activity at 0.6% CHAPS containing 1 M NaCl, and at 1.0% CHAPS containing 0.5 or 0.1 M NaCl. The binding activity in the solubilized fractions did not display a sigmoid curve, as a single peak is appeared, which may have been the result of progressive solubilization. The decrease may have been caused by inactivation at higher detergent concentrations (Fig. 2).

P2 was treated with increasing detergent concentrations at 1M NaCl, and ecdysone binding activity was measured (Fig. 3). Optimal solubilization was achieved at 0.6% CHAPS, yielding 75.1% binding activity relative to specific binding of the crude membrane fractions.

The concept of micelle formation is relevant to solubilization and reconstitution studies of membrane proteins, since it appears that there is some correlation between the ability to form micelles and the concentration of the detergent required for solubilization (Rivnay and Metzger, 1982). CMC, critical miceller concentration, is an important parameter for a given detergent, since at this concentration, the detergent starts to accumulate in the membrane. It is known that the addition of salt dramatically decreases the CMC of charged detergents such as sodium dodecyl sulfate (SDS) (Reynolds and Tanford, 1970; Mukerjee *et al.,* 1970; Chattopadhyay and London, 1984); ionic interactions are reduced with an increase in salt concentration, leading to more effective dissociation of the membrane protein from the non-soluble membrane constituents as well as from other soluble molecules. We found that CHAPS was ineffective in solubilizing ecdysone binding sites from membranes in the absence of NaCl. Therefore, we performed analogous studies with P1 membranes (Fig. 2). The solubilization efficiency of CHAPS increased with increasing salt concentration. CHAPS in the presence of NaCl efficiently solubilizes G-protein-coupled serotonin 1A receptors from bovine hippocampus and from Chinese hamster ovary cells

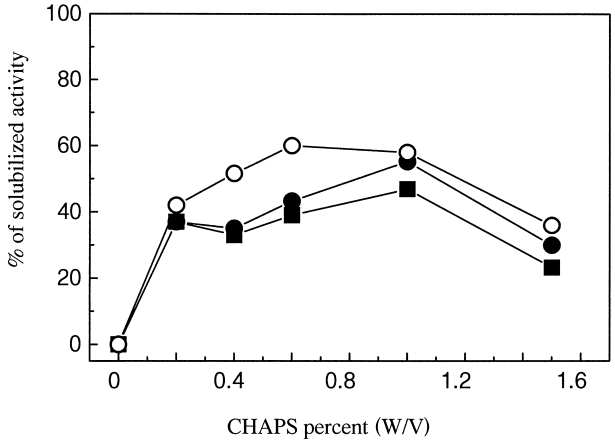

Fig. 2. Solubilization of P1 membranes with different concentrations of CHAPS and NaCl. P1 membranes of ASGs, prepared as described in materials and methods, were suspended in assay buffer to a final protein concentration of 1 mg/ml and treated with combinations of increasing concentrations of CHAPS and NaCl for 60 min at 4°C. Binding activity in the dialyzed supernatants was assayed with 25 nM of [^{3}H]PonA under standard assay conditions. These activities were regarded as solubilized activities. The ordinate is solubilized binding activity shown as the percentage of that in the crude membranes. Open squares, 100 mM NaCl; closed circles, 500 mM NaCl; open circles, 1 M NaCl. Each datum point is the mean of duplicate determinations.

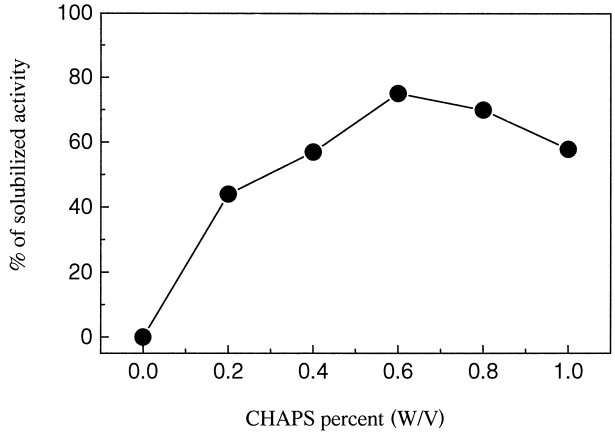

Fig. 3. Solubilization of P2 membranes of ASGs using different concentrations of CHAPS and 1M NaCl. P2 membranes of ASGs, prepared as described in Materials and Methods, were suspended in assay buffer to a final protein concentration of 1 mg/ml and treated with combinations of increasing concentrations of CHAPS and 1M of NaCl for 60 min at 4°C. Binding activity in the dialysed supernatants was assayed with 25 nM of [^{3}H]PonA under standard assay conditions. These activities were regarded as solubilized activities. The ordinate is solubilized binding activity shown as the percentage of that in the crude membranes. Each datum point is the mean of duplicate determinations.

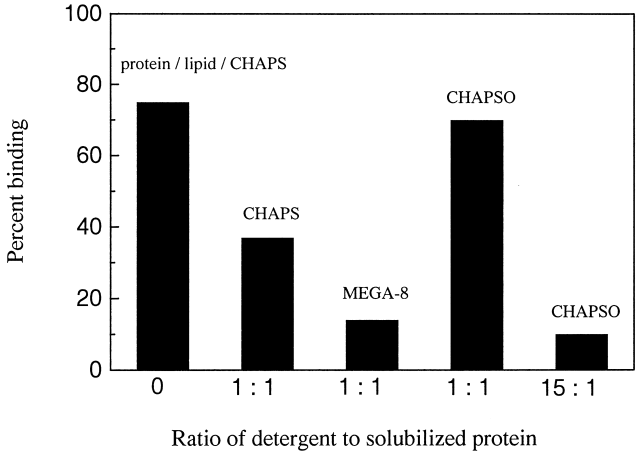

Fig. 4. Dissociation of the mixed micelle complex. To aliquots of dialyzed protein/ lipid/CHAPS mixed micelles (100 μg), CHAPS, MEGA-8, or CHAPSO was added to yield a detergent/protein ratio of 1:1 for the detergents CHAPS, and MEGA-8; 1:1 and 15:1 for CHAPSO. After incubation for 60 min at 4°C, binding activity was measured and is expressed as a percentage of the binding measured in the crude membranes.

(Chattopadhyay and Harikumar, 1996; Chattopadhyay *et al*., 2002, 2004), nicotinic acetylcholine receptors (Schurholz, 1996), dopamine-D2 receptors from synaptosomal membranes of the bovine caudate nucleus (Kidric *et al*., 1984), and the binding sites for gonadotropin-releasing hormone from pituitary plasma membranes (Winiger *et al*., 1983).

Dissociation of the solubilized mixed micelles

Solubilization of membrane proteins is a process in which the proteins and lipids that are held together in the native membrane are suitably dissociated in a buffered detergent solution. The controlled dissociation of the membrane results in the formation of small protein and lipid clusters that remain dissolved in the aqueous solution. Solubilization therefore involves multiple changes in the organization of membranes and depends on a variety of membrane parameters. Accordingly, we next tried to establish experimental conditions to dissociate the protein/lipid/CHAPS mixed micelles we obtained into protein/detergent and lipid/detergent complexes that retained binding activity, so as to decrease the size of the micelles for mEcR protein purification. Different amounts of MEGA-8, CHAPS, and CHAPSO were added to aliquots of the dialyzed protein/lipid/CHAPS mixed micelles to yield detergent/protein ratios of 1:1 and 15:1 (Fig. 4). The binding activity decreased to approximately 40% and 15% at the ratio of 1:1 for CHAPS and MEGA-8, respectively. Binding activity decreased with increasing CHAPSO concentrations from 70% of the original binding activity at the detergent/protein ratio of 1:1 to 8% at the ratio of 15:1. It has been established that an increase in the detergent ratio results in more delipidation of mixed micelles, and that lipid-free protein/detergent complexes are formed at detergent:protein ratios of 10:1 to 20:1 (Hjelmeland and Chrambach, 1984). Accordingly, the observed decrease in binding activity after treatment with MEGA-8 at a 1:1 ratio, CHAPS at a 1:1 ratio, and CHAPSO at a 15:1 ratio (Fig. 4) may have been due to conformational changes in the macromolecule upon substitution of the detergents for the lipid in the microenvironment of the receptor protein.

In conclusion, we defined experimental conditions that allowed solubilization from ASGs membranes of putative membrane ecdysone receptor that retained 75% of its native binding activity, thereby yielding protein/detergent complexes with partial binding activity. This makes it possible to purify membrane-bound ecdysone binding protein.

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