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Partitioning of ecdysteroids using temperature-induced phase separation

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ABSTRACT

The partitioning behaviour of ecdysone and 20-hydroxyecdysone in aqueous two-phase systems was characterized. Primary systems were composed of an ethylene oxide–propylene oxide random copolymer, UCON 50-HB-5100, and dextran T500. The ecdysteroids were first partitioned in a two-phase system with a UCON-rich upper phase and a dextran-rich lower phase. After the phases had separated, the upper phase was removed and isolated in a separate container. This UCON solution was placed in a water bath and the temperature increased above the cloud point of the polymer. This resulted in the formation of a new two-phase system with an upper water–buffer phase and a lower UCON phase. Ecdysteroids partitioned mainly to the final upper water phase in this new two-phase system. Therefore, temperature-induced phase separation could be utilized to recover UCON polymer and obtain ecdysteroids in a water–buffer solution. The partitioning behaviour was manipulated by adding ethanol, sodium chloride or sodium sulphate to the primary two-phase systems. The recovery of ecdysteroids increased when ethanol was added to the system. In a two-phase system with an ethanol concentration of 20%, recovery was 73.6% for ecdysone and 85.6% for 20-hydroxyecdysone.

INTRODUCTION

Partitioning in aqueous two-phase systems is a standard technique for separation and purification of biomolecules [1–4]. Recently, temperature-induced phase separation has been introduced for enzyme purification [5,6]. This technique is possible because of the relatively low cloud point (50°C) of a random copolymer of 50% (w/w) ethylene oxide and 50% (w/w) propylene oxide (UCON 50-HB-5100). UCON is mixed with either dextran or hydroxypropyl starch to form an aqueous two-phase system with an upper UCON-rich phase and a lower phase enriched in either dextran or hydroxypropyl starch. When the target enzyme or other substance partitions to the upper UCON phase, this phase can be removed and the temperature increased above the cloud point of UCON. This results in the formation of a new aqueous two-phase system consisting of an upper water–buffer phase and a lower UCON-rich phase. Enzymes partition strongly to the upper phase of this new system, and the lower UCON phase can be recovered and recycled. The cloud point of UCON can be lowered by changing the ratio of ethylene oxide–propylene oxide groups or by addition of salts such as sodium sulphate or sodium chloride.

Ecdysteroids are a group of polyhydroxylated steroids derived from cholesterol through a variety of metabolic pathways found primarily in the Arthropoda [7,8]. These are phylogenetically old ste-

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roids [9] that are also found in plants and other invertebrate phyla [10,11]. Most ecdysteroids are precursors or metabolites of ecdysone (α-ecdysone: 2β,3β,14α,22*R*,25-pentahydroxy-5-β-cholest-7-en-6-one) and 20-hydroxyecdysone (β-ecdysone), the hormones that regulate the moulting cycle of anthropods [7]. 20-Hydroxyecdysone has also been implicated as a potential sex pheromone in some Crustacea [12–14]. Commercially, ecdysone and 20-hydroxyecdysone are important insecticides [15,16]. Recently these steroids have been detected in the urine of humans parasitized by helminths and nematods [17,18] or suffering from other medical disorders [19]. Consequently, ecdysteroids are medically important in the diagnosis of some pathological conditions in humans [19].

Karlson *et al.* [20] first obtained ecdysones from silkworm pupae, *Bombyx mori*, and described the original extraction and quantification procedures [21]. Two extraction procedures were described. One utilized methanol, butanol and light petroleum (b.p. 65–117°C) in several steps to obtain the desired compounds, while the other applied hot water (80–90°C) and butanol. In each, quantification of the ecdysones was by weight of the residue obtained in the final step after the solvent, methanol or butanol, was evaporated. Verification of the desired compound was obtained through a bioassay that tested its ability to initiate moulting in the pupae of a dipteran fly, *Calliphora erythrocephala* [21]. Since the work of Karlson and Shaaya [21], a few modifications have been made to the extraction procedures [8,11,22,23]. Methanol remains the primary solvent and the desired product is obtained after a number of steps. Detection and quantification of the ecdysones have become more sophisticated but increasingly complex and time-consuming [23–27]. The least complex and most sensitive method is the use of reversed-phase high-performance liquid chromatography (RP-HPLC) combined with UV absorption spectrophotometry [11,24,28–30].

One problem with using two-phase partitioning to purify steroids is that most steroids are insoluble in water. There have been two previous studies published on the partition of steroids [31,32]. Johansson and Joelsson [32] partitioned steroids in two-phase systems composed of polyethylene glycol (PEG), dextran and N,N-dimethylformamide. Shanbhag *et al.* [31] partitioned testosterone, cortisol and oestradiol, both free and bound to hormone receptor proteins, in a two-phase system composed of PEG and dextran. It is of interest to note that, while these steroids are relatively insoluble in water, they were soluble at low concentrations in an aqueous PEG–dextran two-phase system. Ecdysone and 20-hydroxyecdysone are unique among steroids because they are water-soluble. These molecules are identical in structure except for substitution of a hydroxyl group for a hydrogen at position 20 on 20-hydroxyecdysone. This substitution results in different hydrophobicities for these two molecules, with ecdysone being the most hydrophobic.

Aqueous two-phase systems offer an inexpensive and simple method for separation of many biomolecules [33]. A purification scheme for enzymes utilizing temperature-induced phase separation has also been developed [5,6]. By applying this system it is possible to recover the target molecules in a water phase free of polymers without additional ultrafiltration or dialysis steps. Therefore, application of an aqueous two-phase system and temperature-induced phase separation eliminates time-consuming and therefore expensive purification steps previously necessary to remove any organic solvents used in ecdysteroid extraction. In addition, separation using aqueous two-phase systems is a rapid process that does not require any specialized equipment, and the ecdysteroid is obtained in an inert aqueous solution. Therefore, it would be advantageous to develop a method utilizing this system for detection and purification of ecdysone and 20-hydroxyecdysone. However, before such a scheme can be developed, it is necessary to determine how these molecules are distributed in an aqueous two-phase system, and how to manipulate the system to achieve desirable partitioning. With this objective in mind, the partitioning behaviour of these molecules in both an initial UCON–dextran system and a new UCON–water system induced by increasing the temperature was studied. The systems were also modified by adding either salt or ethanol or a combination of these two and observing any effect on the molecules' partitioning.

EXPERIMENTAL

Chemicals

UCON 50-HB-5100 [monobutyl ether of ethyl-

ene oxide (EO)–propylene oxide (PO) random copolymer, EO/PO weight ratio 1:1, M_r 4000] was a kind gift from Union Carbide (New York, NY, USA). Before use in experiments, UCON polymer was ultrafiltered using a Filtron ultrafiltration unit (membrane cut-off 1000 molecular weight) (Filtron Technology Corporation, Clinton, MA, USA) to remove contaminants with light absorption at 280 nm. Dextran T500, M_r 500 000, was obtained from Pharmacia (Uppsala, Sweden). All other biochemicals were of analytical reagent grade.

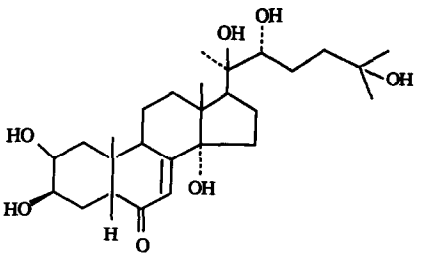

Fig. 2. Structure of 20-hydroxyecdysone (β-ecdysone; compound **2**).

Ecdysteroids

Both ecdysteroids were purchased from Sigma (St. Louis, MO, USA) in crystalline form (>95% purity). α-Ecdysone, $C_{27}H_{44}O_6$, molecular mass 464.6 (EC 3604-87-3) (**1**, Fig. 1), was dissolved in 20% methanol and water. β-Ecdysone, $C_{27}H_{44}O_7$, molecular mass 480.6 (EC 5289-74-7) (**2**, Fig. 2), was dissolved in water.

Ecdysteroid concentration was determined photometrically using a Shimadzu UV-240 double-beam spectrophotometer. The extinction coefficient for α-ecdysone was $\varepsilon_{280} = 5614\ M^{-1}\ cm^{-1}$ and for β-ecdysone $\varepsilon_{280} = 4478\ M^{-1}\ cm^{-1}$ [20].

Two-phase systems and temperature-induced phase separation

All polymer concentrations were calculated as weight percentages. The dextran T500 stock solution was 20% (w/w). The concentration of dextran was determined by polarimetry using an Optical Activity AA-10 automatic polarimeter (Optical Activity, UK) equipped with a sodium lamp set for 589 nm [2]. UCON stock solution was prepared as follows: after ultrafiltration, diluted UCON solution was placed in a water bath at 56°C for 30 min until a lower, concentrated UCON phase and an upper water phase were formed. The lower UCON phase was isolated and the UCON concentration determined using an immersion refractometer with an L1 prism (Carl Zeiss, Germany). This concentrated UCON was diluted to 40% (w/w) and used as stock solution for all experiments.

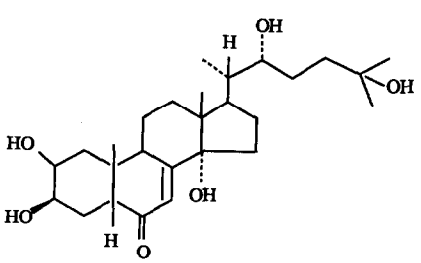

Fig. 1. Structure of α-ecdysone (compound **1**).

Aqueous two-phase systems were prepared from the above stock solutions of polymers in water. Polymer solutions were weighed out and mixed with water, buffer, ecdysteroids and in some cases salt and/or ethanol. Phase systems were separated by centrifugation at 125 *g* for 10 min. The upper UCON-rich phase was removed and isolated in a separate container before being placed in a water bath at 56°C for 15 min. In one set of experiments (systems with 5% or 10% ethanol), sodium sulphate was added to this upper phase to a concentration of 0.1 *M* before the temperature increase. In a second set of experiments, sodium chloride was added to a concentration of either 0.05 *M* (systems with 5% or 10% ethanol) or 0.1 *M* (system with 20% ethanol) before temperature increase. This temperature increase resulted in the formation of a new phase system consisting of an upper water phase and an aqueous lower phase that was enriched in UCON [5,6]. Owing to its high viscosity, this lower UCON-rich phase was diluted by a factor of 10 before analysis, while the upper water-rich phase was analysed without dilution.

Methanol was added to a concentration of 45% to the lower, dextran-rich phase from the first partitioning step. The methanol caused dextran to precipitate out of solution, and the tubes were centrifuged for 15 min at 200 *g*. The methanol supernatant containing the ecdysteroids was removed for analysis, and the dextran pellet was discarded.

Two-phase partitioning of ecdysteroids

The partitioning of ecdysteroids between phases was determined by removing appropriate amounts from the methanol supernatant after dextran precipitation, the upper water phase at 56°C and the lower UCON-rich phase at 56°C, and subjecting them to RP-HPLC analysis (see below, *HPLC analysis*). The concentration in the upper UCON phase from the first partitioning step was calculated to be the sum of the amount of ecdysteroids obtained in the upper and lower phases formed by an increase in temperature. For all partitioning experiments, blank systems were made and samples were removed and injected to determine any interference. The partitioning of ecdysteroids between the two phases is defined by the partition coefficient, K:

$$K = C_t/C_b$$

where C_t and C_b are the concentrations of partitioned substance in the upper and lower phases, respectively, under equilibrium conditions [2]. The distribution ratio, G, is defined as:

$$G = K(V_t/V_b)$$

where V_t and V_b are the volumes of the upper and lower phases, respectively, and therefore G gives the ratio between total amount of ecdysteroid in each phase [2].

HPLC analysis

RP-HPLC was used to determine the concentration of ecdysteroids in all samples [11,24,28–30]. A Waters Delta Pak C_{18} 100-Å, 150 m × 3.9 mm I.D. column (Waters Chromatography Division, Tokyo, Japan) was coupled to a Merck-Hitachi L-6200 pump (Merck-Hitachi, Tokyo, Japan) attached to a Waters Model 441 absorbance detector with a fixed wavelength of 280 nm connected to a potentiometric recorder. Before use, the column was equilibrated with 60% methanol and water. The flow-rate was set at 0.7 ml min^{-1}, and the pressure ranged between 1680 and 1720 p.s.i. The injection volume was set at 0.1 ml.

The elution volume and absorption for ecdysteroids were determined by injecting α-ecdysone (1.0 mg ml^{-1}) and β-ecdysone (0.5 mg ml^{-1}), both separately and in a mixed sample. Elution volumes were 2.4 ml and 2.2 ml, respectively. Standard peak heights were 5.1 cm for 0.1 mg of α-ecdysone and 2.9 cm for 0.05 mg of β-ecdysone, with the detector sensitivity set at 0.2 absorbance units.

Blank injections containing no ecdysteroids were also made with UCON polymer, methanol supernatant from the dextran lower phase, the upper water phase from separation at 56°C and the lower UCON phase from separation at 56°C. None of these solutions eluted at the same volume as the ecdysteroids.

RESULTS AND DISCUSSION

Partitioning of ecdysteroids in a UCON–dextran system

Ecdysone (**1**) and 20-hydroxyecdysone (**2**) were partitioned in a two-phase system composed of 5.0% UCON 50-HB-5100, 4.0% dextran T500, and 0.012 M sodium phosphate buffer, pH 7.0 (Table I). The K value, which reflects the relative affinity of the compound for the two phases, is affected by many parameters, including hydrophobic interactions [2,34]. Since UCON is more hydrophobic than dextran, different partitioning behaviour can be predicted for the two ecdysteroids, depending on their hydrophobicity [35]. This trend was not reflected in their partitioning in the primary UCON–dextran system, with K = 1.12 for ecdysone and K = 1.30 for 20-hydroxyecdysone (Table IA). However, when the upper UCON phase was removed and the temperature increased to 56°C, the more hydrophobic ecdysone partitioned much more strongly to the lower UCON phase (K = 0.59) than 20-hydroxyecdysone (K = 1.34), which was enriched in the upper water phase.

Addition of salt to the two-phase systems increases hydrophobic interactions [36]. To observe this effect on the partitioning of ecdysone and 20-hydroxyecdysone, sodium chloride was added to the above system to a concentration of 0.04 M. As expected, this increased the affinity of ecdysone for the upper UCON-rich phase (K = 1.21), while the partition coefficient for more hydrophilic 20-hydroxyecdysone decreased to 1.14 (Table IB). Partitioning at 56°C between water and UCON phases for ecdysone was relatively unchanged (K = 0.60), while for 20-hydroxyecdysone the K value decreased to 1.04.

G values, which depend on the volume ratio between upper and lower phases, were also affected by addition of salt. This was partly the result of a slight

TABLE I

PARTITIONING OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 5.0% UCON 50-HB-5100, 4.0% dextran T500, 1.0 mg of α-ecdysone, 0.5 mg of β-ecdysone and 0.012 *M* sodium phosphate buffer, pH 7.0. *K* and *G* values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. (A) Primary system wihout sodium chloride added; (B) primary system with 0.04 *M* sodium chloride added. *Y* = percentage yield of ecdysones in the water phase. (A) Volume ratio (V_t/V_b) at 22°C = 1.8, at 56°C = 11.3; (B) volume ratio (V_t/V_b at 22°C = 1.9, at 56°C = 8.25.

Compound	*K* (22°C)	*G* (22°C)	*K* (56°C)	*G* (56°C)	*Y*
(A) Without sodium chloride					
α-Ecdysone	1.12	2.03	0.59	6.71	58.4
β-Ecdysone	1.30	2.37	1.34	15.16	65.9
(B) With sodium chloride					
α-Ecdysone	1.21	2.31	0.60	4.99	58.0
β-Ecdysone	1.14	2.17	1.04	8.56	61.3

increase in the volume of UCON phase from 0.3 ml to 0.4 ml when sodium chloride was added to 0.04 *M*. The *G* value for 20-hydroxyecdysone decreased from 15.16 to 8.56 and for ecdysone from 6.71 to 4.99.

Effect of ethanol on the partitioning of ecdysteroids

Ecdysone and 20-hydroxyecdysone were partitioned in a two-phase system composed of 6.0% UCON, 5.0% dextran T500 and 0.012 *M* sodium phosphate buffer, pH 7.0, and either 5.0%, 10.0% or 20.0% ethanol (Tables II and III). Since UCON is more hydrophobic than dextran, ethanol can be expected to partition preferentially to the upper UCON phase, thereby further increasing the hydrophobicity of this phase. After the UCON and dextran phases had separated, the upper UCON phase was isolated in a separate container and sodium chloride was added to this phase to a concentration of 0.05 *M* (Table II). For the 20.0% ethanol system sodium chloride was added to this upper phase to 0.1 *M* (Table III). The addition of salt was necessary in order to obtain phase separation when ethanol had been included in the original two-phase system. This UCON–water–salt solution was placed in a water bath at 56°C for 15 min to achieve formation of a new two-phase system.

In Fig. 3 the log *K* values at 22°C for ecdysone

TABLE II

PARTITIONING OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 6.0% UCON 50-HB-5100, 5.0% dextran T500, 1.0 mg of α-ecdysone, 0.5 mg of β-ecdysone and 0.012 *M* sodium phosphate buffer, pH 7.0; 0.05 *M* sodium chloride was added to the aqueous UCON phase prior to increasing the temperature. *K* and *G* values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. (A) Primary system with 5.0% ethanol added; (B) primary system with 10.0% ethanol added. *Y* = percentage yield of ecdysones in the water phase. (A) Volume ratio (V_t/V_b) at 22°C = 3.25, at 56°C = 5.5; (B) Volume ratio (V_t/V_b) at 22°C = 2.60, at 56°C = 4.8.

Compound	*K* (22°C)	*G* (22°C)	*K* (56°C)	*G* (56°C)	*Y*
(A) With 5% ethanol					
α-Ecdysone	1.28	4.15	0.17	0.95	39.3
β-Ecdysone	1.04	3.38	0.39	2.17	52.8
(B) With 10% ethanol					
α-Ecdysone	1.79	4.72	0.43	2.07	55.6
β-Ecdysone	1.34	3.54	0.68	3.30	59.9

TABLE III

PARTITIONING OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 6.0% UCON 50-HB-5100, 5.0% dextran T500, 1.0 mg α-ecdysone, 0.5 mg of β-ecdysone, 20.0% ethanol and 0.012 *M* sodium phosphate buffer, pH 7.0; 0.1 *M* sodium chloride was added to the aqueous UCON phase prior to increasing the temperature. *K* and *G* values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. *Y* = percentage yield of ecdysones in the water phase. Volume ratio (V_t/V_b) at 22°C = 4.0, at 56°C = 6.3.

Compound	*K* (22°C)	*G* (22°C)	*K* (56°C)	*G* (56°C)	*Y*
α-Ecdysone	3.01	12.02	0.62	3.94	73.6
β-Ecdysone	3.18	12.72	1.91	12.09	85.6

and 20-hydroxyecdysone obtained from Tables IA, IIA, IIB and III have been plotted as a function of ethanol concentration. For ecdysone log *K* increased from 0.05 with no ethanol to 0.48 with 20% ethanol. Log *K* for 20-hydroxyecdysone exhibited a different tendency, first decreasing with 5% ethanol (log *K* = 0.02), then increasing to 0.50 when 20% ethanol was included in the system. The different partitioning behaviour of ecdysteroids with 5% ethanol most likely reflects their different hydrophobicities. While ecdysone was drawn more strongly into the hydrophobic UCON phase, 5% ethanol had much less effect on 20-hydroxyecdysone, even pushing it slightly more into the hydrophilic dextran phase. However, as the concentration of ethanol was raised this effect diminished, with both

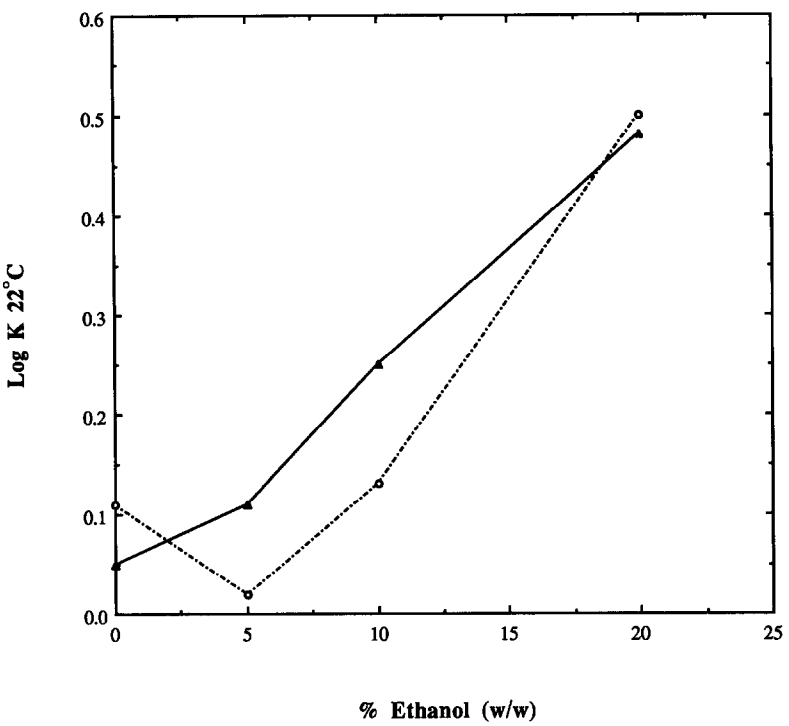

Fig. 3. Effect of ethanol concentration on the partioning at 22°C of α-ecdysone (▲) and β-ecdysone (○). Two-phase systems containing ethanol were composed of 6.0% UCON 50-HB-5100, 5% dextran T500 and 0.01 *M* sodium phosphate buffer, pH 7.0. The system without ethanol was 5.0% UCON 50-HB-5100, 4.0% dextran T500 and 0.01 *M* sodium phosphate buffer, pH 7.0. Data were obtained from Tables IA, IIA, IIB and III.

ecdysteroids being drawn increasingly into the upper UCON-rich phase.

In Fig. 4 log K values from Tables IB, IIA, IIB and III for the partition of ecdysone and 20-hydroxyecdysone at 56°C have been plotted as a function of ethanol concentration. At 56°C it is harder to determine the effect of ethanol on the partitioning of ecdysteroids owing to addition of salt to this upper phase. Partitioning of both ecdysteroids at 56°C to the UCON-rich phase increased dramatically with the addition of 5% ethanol (log K = −0.77 for ecdysone and −0.41 for 20-hydroxyecdysone), while further addition of ethanol increasingly pulled them into the upper water–ethanol–buffer phase. At 20% ethanol the partition coefficient at 56°C for 20-hydroxyecdysone was higher (log K = 0.28) than when no ethanol had been added (log K = 0.02). For ecdysone the two values were roughly equal, with log K = −0.22 when no ethanol was present and log K = −0.21 when 20% ethanol was included in the system. This result was consistent throughout the experiments, with the more hydrophobic ecdysone displaying a greater affinity for the UCON phase than 20-hydroxyecdysone.

The yield of ecdysteroids was calculated from the amount of ecdysteroid recovered in the upper water phase after separation at 56°C. For the phase system without ethanol or salt added, the yield of ecdysone was 58.4% and of 20-hydroxyecdysone 65.9% (Table IA). Addition of sodium chloride to the system lowered the yield of ecdysone to 58.0% and of 20-hydroxyecdysone to 61.3% (Table IB). When 5% ethanol was included in the system the yields of ecdysone and 20-hydroxyecdysone were 39.3% and 52.8%, respectively (Table IIA). Increasing ethanol to 10% increased the yield for ecdysone to 55.6% and for 20-hydroxyecdysone to 59.9% (Table IIB). The best yield of both ecdysones

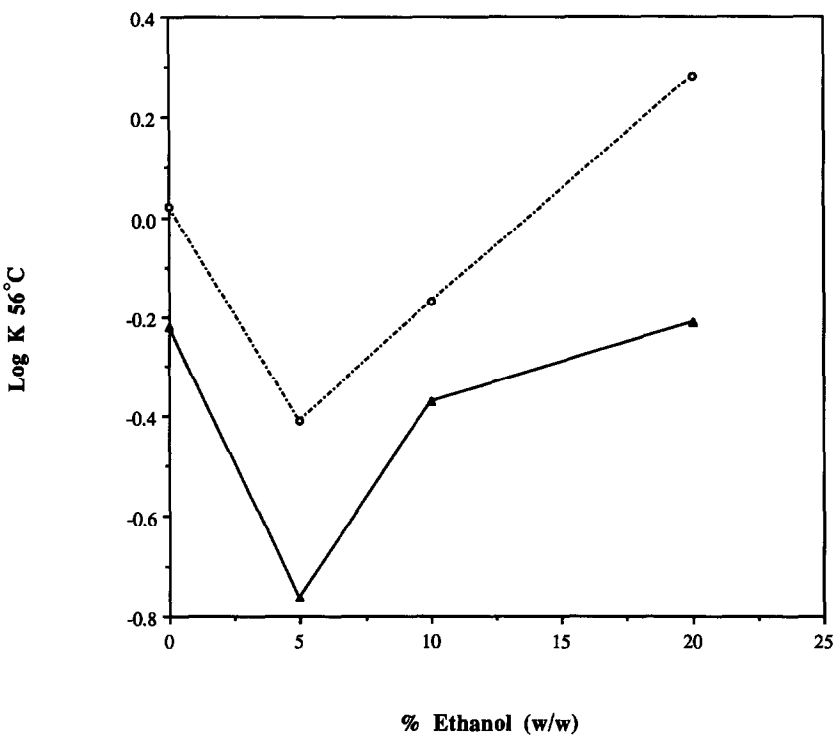

Fig. 4. Effect of ethanol concentration on the partitioning at 56°C of α-ecdysone (▲) and β-ecdysone (○). Primary systems were the same as described in Fig. 3. Sodium chloride was added to the removed upper phase from primary separation before increasing the temperature. The salt concentrations were as follows: 0.04 M, upper phase from primary system with no ethanol; 0.05 M, upper phase from primary systems with 5% and 10% ethanol; 0.1 M, upper phase from primary system with 20% ethanol. Data were obtained from Tables IB, IIA, IIB and III.

was obtained in a system which included 20% ethanol where recovery for ecdysone was 73.6% and for 20-hydroxyecdysone 85.6% (Table III).

Addition of sodium sulphate to two-phase systems containing ethanol

Adding a salt containing a divalent anion, sodium sulphate (0.1 *M*), to the upper UCON-rich phase before increasing the temperature had an effect on the partitioning of ecdysteroids. Partitioning experiments were carried out in systems containing either 5% or 10% ethanol (Table IV). In the system with 5% ethanol, *K* at 56°C was 0.25 for ecdysone and 0.46 for 20-hydroxyecdysone. *K* values at 56°C for the system with 10% ethanol were lower (*K* = 0.26 for ecdysone and 0.52 for 20-hydroxyecdysone) than in a system with 10% ethanol and sodium chloride (*K* = 0.43 for ecdysone and 0.68 for 20-hydroxyecdysone, Table IIB). These results reflect an increased hydrophilicity of upper water–salt phases at 56°C when sodium chloride was replaced with sodium sulphate. The yield of ecdysteroids was also fairly low in systems containing sodium sulphate. With 5% ethanol the yield was 47.7% for ecdysone and 56.2 for 20-hydroxyecdysone. Increasing the ethanol concentration to 10% had little effect on total recovery of ecdysteroids, with yields of 46.4% for ecdysone and 58.6% for 20-hydroxyecdysone.

TABLE IV

PARTITIONING AT 56°C OF α-ECDYSONE AND β-ECDYSONE (20-HYDROXYECDYSONE)

Primary phase systems: 6.0% UCON 50-HB-5100, 5.0% dextran T500, 1.0 mg of α-ecdysone, 0.5 mg of β-ecdysone and 0.012 *M* sodium phosphate buffer, pH 7.0; 0.1 *M* sodium sulphate was added to the aqueous UCON phase prior to increasing the temperature. *K* and *G* values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. (A) Primary system with 5.0% ethanol added; (B) primary system with 10.0% ethanol added. *Y* = percentage yield of ecdysones in the water phase. (A) Volume ratio (V_t/V_b) at 56°C = 5.3; (B) volume ratio (V_t/V_b) at 56°C = 4.4.

Compound	*K* (56°C)	*G* (56°C)	*Y*
(A) With 5% ethanol			
α-Ecdysone	0.25	1.35	47.7
β-Ecdysone	0.46	2.46	56.2
(B) With 10% ethanol			
α-Ecdysone	0.26	1.16	46.4
β-Ecdysone	0.52	2.32	58.6

Purification scheme for ecdysone and 20-hydroxyecdysone

The results obtained in partitioning these ecdysteroids show that it would be possible to purify them using temperature-induced phase separation. A purification scheme for enzymes using this technique has already been developed [5,6]. In these studies it has been shown that in an initial UCON–dextran or UCON–hydroxypropyl starch phase system most proteins, cell particles and other cell debris partition strongly to the lower phase, leaving an upper UCON phase with little contamination. Based on these findings, it can be expected that when a cell homogenate containing ecdysteroids is added to a UCON–dextran or UCON–hydroxypropyl starch two-phase system the ecdysteroids will partition to the upper UCON-rich phase, while most proteins and other macromolecules will partition to the lower dextran-rich or hydroxypropyl starch-rich phase. Also, addition of ethanol to the phase system should increase this effect, as increased hydrophobicity of upper UCON-rich phase would push hydrophilic biomolecules even more into the lower phase at 22°C and draw the hydrophobic ecdysteroids more into the UCON upper phase. This will result in even greater purification of ecdysteroids. The lower dextran or hydroxypropyl starch phase goes to waste. The upper UCON phase is removed and placed in a water bath at 56°C for 15 min. This temperature increase forms a new two-phase system with an upper water–buffer phase and a lower, highly viscous UCON phase. Ecdysteroids which are recovered in this water phase can be used without further purification. The lower UCON phase is recovered and can be used for future extractions. A system composed of 6.0% UCON 50-HB-5100, 5.0% dextran T500, 20% ethanol and 0.012 *M* sodium phosphate buffer, pH 7.0, shows greatest promise as a possible purification technique for these ecdysteroids (Table III). *K* values were the highest observed at both 22°C and 56°C. The percentage recovery (yield) in the final water–buffer phase was also very high, 73.6% for ecdysone and 85.6% for 20-hydroxyecdysone. The present purification techniques use high concentrations of

organic solvents and repeated extractions, with normal yields of 50–80% [25,28]. However, this study shows that a high yield and purity of ecdysteroids can be achieved by using an aqueous two-phase system combined with temperature-induced phase separation.

CONCLUSIONS

It is possible to partition water-soluble ecdysteroids in an aqueous two-phase system composed of UCON 50-HB-5100 and dextran T500. The use of temperature-induced phase separation allows these molecules to be recovered in an aqueous phase composed of water and buffer that is free of polymers or other contaminants. Since the ecdysteroids are recovered in a water–buffer phase virtually free of contaminants or organic solvents, it should be possible to use them without further purification. The high level of ecdysteroids recovered, 73.6% and 85.6% for α-ecdysone and β-ecdysone, respectively, would allow their detection even when present in small quantities. For these reasons, the use of aqueous two-phase partition combined with temperature-induced phase separation offers great potential as either an analytical or preparative technique for ecdysteroids.

The partitioning behaviour of ecdysteroids in both the primary phase system and in the new temperature-induced system can be manipulated by addition of salt or ethanol. Because of their different hydrophobicities, addition of a small amount of salt or ethanol had a different effect on the partitioning behaviour of ecdysone and 20-hydroxyecdysone. The distribution of the molecules seemed to be influenced by hydrophobic interactions with the upper UCON-rich phase in the primary system and with the lower UCON-rich phase at 56°C. In almost all instances, the more hydrophobic ecdysone partitioned more strongly to the UCON-rich phases than did 20-hydroxyecdysone.

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