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Utilization of temperature-induced phase separation for the purification of ecdysone and 20-hydroxyecdysone from spinach

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Abstract

An aqueous solution of the ethylene oxide–propylene oxide random copolymer UCON 50-HB-5100 was successfully used to extract ecdysone and 20-hydroxyecdysone from the common spinach plant, *Spinacia oleracea*. The UCON spinach extract was mixed with a hydroxypropyl starch Reppal PES 200 solution and allowed to form an aqueous two-phase system. After the polymers separated cell debris, proteins and other comtaminants partitioned to the lower Reppal phase and ecdysone and 20-hydroxyecdysone partitioned to the upper UCON phase. The UCON phase was isolated and subjected to a temperature increase to 56°C which induced phase separation between UCON and water. Ecdysone and 20-hydroxyecdysone partitioned between the UCON phase and the water phase at concentrations determined by their degree of hydrophobicity. The less hydrophobic 20-hydroxyecdysone had a greater affinity for the water-rich phase than did ecdysone. Due to the larger volume of the water phase both ecdysteroids were obtained in this phase at 56°C with yields higher than 80%. With 20% ethanol in the primary system recovery was 88.7% for ecdysone and 91.2% for 20-hydroxyecdysone. Results indicate that aqueous two-phase partitioning coupled with temperature-induced phase separation is a quick, easy and inexpensive bench-top technique for extracting and purifying ecdysteroids from raw material. This technique can also be readily up-scaled for commercial use.

1. Introduction

Ecdysone and 20-hydroxyecdysone, hormones that regulate molting cycles of arthropods, are also found in some plants [1,2]. Both are of commercial interest as insecticides [3–6] and indicators of helminth and nematod parasitism and other medical disorders in humans [7–10]. Both molecules are steroids [1,11], but soluble in water. Their structures differ only in the substitution of a hydroxyl group in 20-hydroxyecdysone for a hydrogen (in ecdysone) on the C-20 position. This substitution changes the hydrophobicity of the two molecules, with ecdysone being the more hydrophobic.

Extraction and recovery of ecdysone, 20-hydroxyecdysone and other ecdysteroids from raw material, *e.g.*, insects and plants, follow classic protocols originally developed by Karlson and Shaaya [12]. Generally, one or several non-polar solvents (*e.g.*, methanol, butanol, light petroleum, etc.) and/or hot water (80–90°C) are used to extract the desired molecules. The molecules are recovered in a residue obtained after the evaporation of the solvent in the final extraction step. Quantification is determined from the mass of this residue and verification is obtained in a bioassay that determines the ability of ecdysone

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and 20-hydroxyecdysone to induce molting of dipteran fly, *Calliphora erythrocephala*, pupae [12]. Although the original extraction procedures have received little or no improvements and methanol remains the solvent of choice [13–16], detection and quantification of ecdysteroids have become more sophisticated [16–23]. Presently, extraction, detection, quantification and recovery procedures for the ecdysteroids are time consuming and require costly, sophisticated chromatographic equipment. Nevertheless, without additional purification steps, desired compounds are contaminated with solvent residues which can have lethal and/or sublethal effects on animals.

Aqueous two-phase partitioning systems, widely used to separate and purify biomolecules, are usually composed of aqueous solutions of two biologically inert and immiscible polymers, *e.g.*, polyethylene glycol (PEG) (upper phase) and dextran or hydroxypropyl starch (lower phase) [24–27]. These systems have gained widespread use for separation of biomolecules, cell particles and cells. In the recent development of temperature-induced phase separation the ethylene oxide–propylene oxide random copolymer of UCON 50-HB-5100 (UCON) had been used instead of PEG in phase systems with dextran or hydroxypropyl starch. The upper UCON-rich phase is removed and isolated in a separate container which is then subjected to increased temperature above the cloud point of UCON. The temperature-induced phase separation results in the formation of a UCON-rich and a water-rich phase. The phase separation can be made to occur at biologically favorable temperatures (37–56°C) which allows purification of biomolecules to a water-rich phase free of polymers [28,29]. The partition behavior of authentic ecdysone and 20-hydroxyecdysone in aqueous two-phase systems (UCON–dextran primary system) combined with temperature-induced phase separation was successfully determined [30]. Ecdysone and 20-hydroxyecdysone, because of their hydrophobicity, partitioned mainly to the upper UCON-rich phase in the primary systems. When temperature was increased on the isolated UCON-rich phase, above the cloud point of UCON (50°C), the two ecdysteroids partitioned between the water-rich phase and the UCON phase at concentrations determined by their degree of hydrophobicity [30].

An aqueous two-phase system composed of the random copolymer UCON 50-HB-5100 (upper phase) and Reppal PES 200 (Reppal; hydroxypropyl starch) (lower phase) was tested in an attempt to obtain ecdysone and 20-hydroxyecdysone from raw material. The use of Reppal in the lower phase, rather than dextran, appeared more desirable because of its lower affinity for hydrophobic molecules and lower cost. In this project we attempted to (1) extract ecdysteroids from raw material using a UCON solution rather than non-polar solvents described in classic protocols [12], (2) develop an UCON–Reppal aqueous two-phase system that would specifically partition ecdysone and 20-hydroxyecdysone to the upper hydrophobic phase, and (3) allow us, after the primary partitioning step, to isolate the ecdysteroids in a non-polymer water-rich solution by subjecting the UCON-rich phase to temperature-induced phase separation.

Although best known as hormones that mediate the molting cycle in insects and other arthropods, concentrations of ecdysone and 20-hydroxyecdysone in plants are more predictable and far exceed that found in insects by several orders of magnitude [1,11]. Consequently, because of its availability and ease of culturing, our choice of raw material was the common spinach plant, *Spinacia oleracea* [31].

2. Experimental

2.1. Chemicals

UCON 50-HB-5100 [ethylene oxide (EO)–propylene oxide (PO) random copolymer, EO–PO ratio 1:1, M_r 4000] was a kind gift from Union Carbide (New York, NY, USA). Prior to use, UCON was purified by extraction into methylene chloride. Reppal PES 200 [hydroxypropyl starch, M_r 200 000] was a kind gift from Reppe (Växjö, Sweden). Ecdysone (M_r 464.6, EC 3604-87-3) was dissolved in methanol–water

(20:80) and 20-hydroxyecdysone (M_r 480.6, EC 5289-74-7) was dissolved in water prior to use. Both ecdysteroids were purchased in crystalline form (>95% purity) from Sigma (St. Louis, MO, USA). All other chemicals were of analytical-reagent grade.

2.2. UCON Spinach extract

Spinach plants were grown from seed in a greenhouse at ambient temperature, light intensity and photoperiod for 46 days before leaves were removed (194 g fresh mass), homogenized (Waring Blender) and sonicated (Branson Cell Disrupter) for 15 min in a solution comprised of 100 g UCON and 294 g phosphate buffer (P.B.) (0.1 *M*, pH 7.0). Ecdysteroid concentrations in spinach leaves is fairly constant at 65 μg/g fresh mass in 44–76-day-old plants [31]. Extraction was allowed to proceed for 72 h at 4.0°C. The extract was then centrifuged at 17 000 *g* for 20 min at 4.0°C to remove plant particulates. Based on fluid volume of the extract after centrifugation (394 ml), UCON concentration was 25.4%.

2.3. Primary aqueous two-phase partition system (primary system)

The phase diagram for the system UCON–Reppal–water (Fig. 1) was determined as described previously [28]. All polymer concentrations were calculated as mass percentages. The UCON spinach extract was mixed with an aqueous stock solution of Reppal (21%, w/w) to a final concentration of 11.0% UCON and 7.5% Reppal. Two duplicate experimental primary phase systems were tested, one with 20% ethanol added and the other without ethanol. The primary phase systems were kept at room temperature (22°C). Phase systems were separated by centrifugation at 125 *g* for 10 min after which the UCON-rich upper phase was removed and isolated in a separate container. The lower, Reppal-rich phase, was treated with methanol–water (45:55) to precipitate the Reppal and concentrate any ecdysones in the supernatant. These tubes were centrifuged at 200 *g* for 15 min before the supernatant was analyzed.

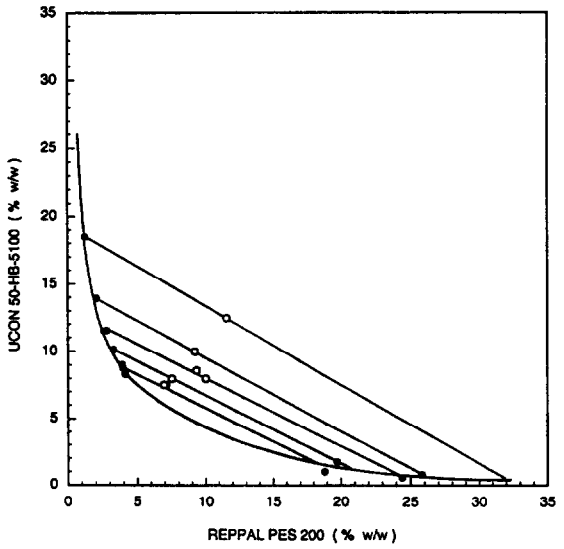

Fig. 1. Phase diagram for UCON 50-HB-5100 (M_r 4000), Reppal PES 200 (hydroxypropyl starch, M_r 200 000) and water at 22°C. ● = points obtained by tritration [28,31] or analysis of separate phases; ○ = points obtained by mixing of polymers.

2.4. Temperature-induced phase separation

Containers with upper UCON-rich phase were placed in a water bath at 56°C for 20 min to allow temperature-induced phase separation to occur. The upper water–buffer phase was removed and isolated prior to analysis. Because of increased viscosity, the UCON phase was diluted by a factor of 10 prior to analysis.

2.5. Detection and analysis

Reversed-phase high performance liquid chromatography (RP-HPLC) coupled with UV spectrophotometry was used to determine presence and concentrations of ecdysone and 20-hydroxyecdysone in all samples. A Waters Delta Pak C_{18} 100-Å, 150 mm × 3.9 mm I.D. column (Waters Chromatography Division, Tokyo, Japan) was coupled to a Bio-Rad HPLC pump attached to a Perkin-Elmer LC75 UV-absorbance detector (wavelength preset at 254 nm) connected to a potentiometric recorder. The column was equilibrated before use with methanol–water (40:60). Flow-rate of the methanol–water (40:60)

medium was 0.7 ml min^{-1} and pressures ranged from 95–114 kg $(cm^2)^{-1}$. The injection volume was 0.1 ml. Samples were filtered through a 0.2-μm Gelman syringe filter before injection.

Elution time and absorption for ecdysteroids was determined by injecting known amounts of authentic ecdysone (0.042 mg ml^{-1}) and 20-hydroxyecdysone (0.05 mg ml^{-1}) separately and in mixed samples. Elution times were 11.0 min for 20-hydroxyecdysone and 23 min for ecdysone. Peak heights were used to calibrate dose–response curves because of the narrowness of the traces and to allow comparison with previous work [30]. Peak heights for 20-hydroxyecdysone and ecdysone standards were, respectively, at 3.6 cm and 1.7 cm with sensitivity set at 0.2 absorption units.

Blank injections with no ecdysteroids were made with UCON polymer, methanol supernatant of the Reppal lower phase, the upper water phase after separation at 56°C and the lower UCON phase after separation at 56°C.

2.6. Quantification

Degree of partitioning of ecdysteroids between the various phases was determined by removing known quantities from the methanol supernatant after Reppal precipitation, the upper water phase at 56°C and UCON phase at 56°C and subjecting them to RP-HPLC analysis. Concentration in the upper UCON phase from the primary partition step was calculated to be the sum of the ecdysteroids obtained in the upper and lower phases produced by an increase in temperature. Blank systems were made for all partitioning experiments and samples were removed and injected to determine any interference. Results are defined by the partition coefficient, $K = C_t/C_b$, where C_t and C_b are the concentrations of partitioned substances in the upper and lower phases, respectively, under equilibrium conditions [25] and by the distribution ratio, $G = K(V_t/V_b)$, where V_t and V_b are the volumes of the upper and lower phases, respectively. G gives the ratio between the total amounts of ecdysteroid in each phase [25].

3. Results and discussion

A 25% aqueous solution of UCON was effective in extracting ecdysone and 20-hydroxyecdysone from plant material. Results compare favorably with those obtained by utilizing classic extracting techniques [13,31]. An average of 73.4 μg/g fresh mass (f.m.) (68.8–83.3 μg/g f.m.) of 20-hydroxyecdysone was obtained from 46-day-old spinach leaves using UCON solution. Concentrations of ecdysone in the UCON spinach extract were an order of magnitude less, 2.4–5.0 μg/g f.m. Total ecdysteroid concentration obtained using UCON extraction is slightly more than that realized by Grebenok *et al.* [31] with standard methanol extraction. However, quantitative comparisons are dubious without considering the biological and environmental conditions that influence ecdysteroid amounts in spinach plants.

A phase diagram for the system UCON–Reppal–water at 22°C was constructed (Fig. 1). This was done in order to select suitable polymer concentrations for the primary aqueous two-phase system. The phase diagram is relatively similar to the phase diagram for the system UCON–Dextran T500–water [28]. Phase separation is obtained at slightly higher polymer concentrations because of the lower molecular mass (200 000) of Reppal compared with Dextran T500 (500 000) [26].

Once extraction was completed and the suspension was centrifuged, the primary phase system was easily constructed by mixing an aqueous solution of Reppal into the supernatant to obtain the desired polymer concentrations. The purification scheme is shown in Fig. 2. Phase separation occurs within 10–20 min, or more rapidly if the systems are centrifuged. Cell debris, proteins and other contaminants partitioned to the lower Reppal-rich phase. Chlorophylls were, for the most part, removed during the initial centrifugation. Those that remained partitioned to the lower Reppal-rich phase. Based on the tint of the upper UCON-rich phase, which was extremely pale, chlorophylls appeared to remain attached to membranes and/or chloroplast (cell debris). Coloration in the Reppal-rich

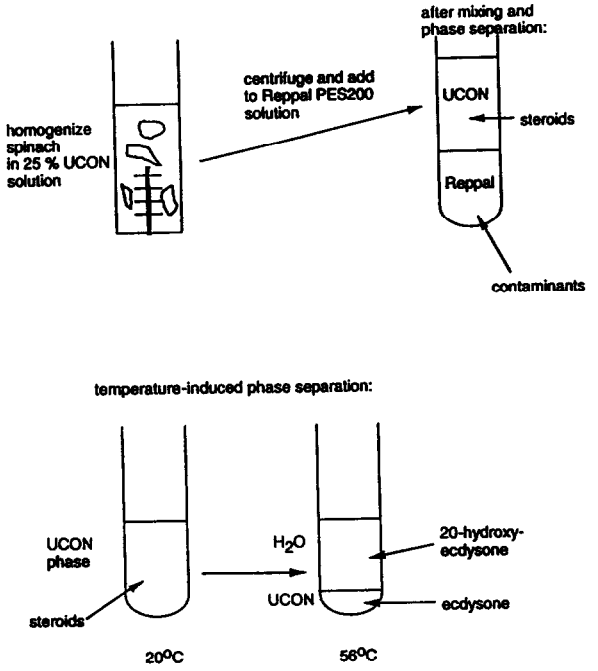

Fig. 2. Scheme for the purification of ecdysteroids from spinach leaves using aqueous two-phase partitioning and temperature-induced phase separation.

phase was noticeable. The ecdysteroids partitioned very strongly to the upper UCON-rich phase with partition coefficients of 50.3 for ecdysone and 30.6 for 20-hydroxyecdysone (Table 1).

After phase separation in the primary aqueous two-phase system the upper UCON-rich phase is removed and isolated in a separate container (Fig. 2). This is then subjected to increased temperature above the cloud point of UCON (50°C). The temperature-induced phase separation results in the formation of a UCON-rich and a water-rich phase. The phase separation was induced at 56°C (Fig. 2). The greater hydrophobicity of ecdysone caused it to partition more to the UCON-rich phase ($K = 0.51$) in systems not containing ethanol, while 20-hydroxyecdysone partitioned to the water-rich phase ($K = 2.16$, Table 1). Ecdysone and 20-hydroxyecdysone could both be recovered in the water-rich phase at yields of 80.5 and 93.8%, respectively, calculated for the original amounts in the spinach extract. The high recovery of the hydrophobic ecdysone (80.5%) in the water phase, in spite of the unfavorable partition coefficient, is due to the larger volume of the water phase relative to the UCON phase. The volume ratio water phase–UCON phase was 8.4 and the G value, which determines the yield, was 4.28 for ecdysone and 18.1 for 20-hydroxyecdysone (Table 1).

Manipulation of the hydrophobicity of aqueous two-phase systems with salts and/or solvents improves their specificity [24–30]. Addition of 20% ethanol to the primary systems containing authentic compounds yielded 73.6% ecdysone and 85.6% 20-hydroxyecdysone of the original amount in the water-rich phase after temperature-induced phase separation [30]. The extraction and recovery of these ecdysteroids from spinach substantiates these results (Table

Table 1
Partitioning of ecdysone and 20-hydroxyecdysone from spinach

Compound	System	K (22°C)	G (22°C)	K (56°C)	G (56°)	Y
Ecdysone	1	50.3	159	0.51	4.28	80.5
	2	>100	>100	1.74	7.0	88.7
20-Hydroxyecdysone	1	30.6	94.5	2.16	18.1	93.8
	2	>100	>100	1.94	11.7	91.2

Primary phase systems: 11% UCON 50-HB-5100 spinach extract, 7.5% Reppal PES 200 and 0.1 M sodium phosphate buffer, pH 7.0. System 1 did not contain ethanol and system 2 contained 20% ethanol. K and G values at 56°C are for partitioning between the water and UCON phases formed by increasing the temperature. Volume ratios (V_t/V_b) at 22°C and 56°C, respectively: system 1, 3.0 and 8.4 and system 2, 3.8 and 5.0. Y = percentage yield of ecdysteroids in the water phase. All values given are mean values from duplicate experiments.

1). Addition of 20% ethanol to the primary phase systems increased the hydrophobicity of the upper UCON-rich phase and forced both ecdysteroids to completely partition into the upper phase ($K > 100$, Table 1). The ethanol affected also the partitioning of the hydrophobic ecdysteroid, ecdysone, between the water and UCON phases formed by raising the temperature. Ecdysone was partitioned to the water-phase in systems containing ethanol. The K value was 1.74 compared with 0.51 obtained in systems without ethanol (Table 1). This can be explained by the increased solubility of ecdysone in the water phase due to the presence of ethanol. The addition of ethanol had little effect on the partitioning of the less hydrophobic 20-hydroxyecdysone. K values for the partitioning between water and UCON phases in systems with ethanol and without were 1.94 and 2.16, respectively. For systems containing ethanol the yields of ecdysone and 20-hydroxyecdysone in the final water phase were 88.7 and 91.2%, respectively (Table 1). Thus, the addition of ethanol increased the recovery of ecdysone in the water phase from 80 to 89%, while the recovery of 20-hydroxyecdysone was kept at more than 90%.

The purification scheme using phase systems without ethanol is shown in Fig. 2. In the last step ecdysone is partitioned to the UCON phase and 20-hydroxyecdysone to the water phase after temperature increase. This is in agreement with results from a recent study on the partitioning of carboxylic acids in UCON–water phase systems [32]. It was found that carboxylic acids of increasing hydrophobicity were, to an increasing degree, partitioned to the UCON-rich phase. Thus, the different partitioning of the two ecdysteroids in the UCON–water system can be explained by the difference in hydrophobicity between the two compounds. It should be pointed out that the system is very sensitive and can detect very small differences in hydrophobicity. In this case only one hydrogen atom in ecdysone has been substituted for one hydroxyl group in 20-hydroxyecdysone. In the UCON–water system it should be possible to utilize the separation based on hydrophobicity for a further purification of the ecdysteroids. After the temperature-induced phase separation both ecdysteroids could be obtained in a water phase virtually free of polymer (Table 1). For the hydrophobic ecdysone this was made possible by having a large volume ratio so that more than 80% of the ecdysteroid was obtained in the water phase in spite of an unfavorable partition coefficient. Alternatively, by adding 20% ethanol in the primary system the partition coefficient of ecdysone was changed in the second phase system, and this ecdysteroid was partitioned to the final water phase with a yield of 89%. However, the ethanol addition removed the difference in the partitioning for the two ecdysteroids and both were partitioned to the same extent to the water phase. Thus, ethanol addition has the advantage that both ecdysteroids are obtained in the water phase with high yield and the drawback that it is not possible to exploit a difference in ecdysteroid partitioning for further purification.

Ecdysone has not previously been reported in spinach. Its presence was verified with RP-HPLC co-chromatography with authentic compound. Additionally, heights of elution peaks generated by presence of ecdysone and 20-hydroxyecdysone were increased when the UCON spinach extract was spiked with authentic compounds. No further verification of ecdysone was preformed.

Polypodine B (5β,20-dihydroxyecdysone) is also an ecdysteroid commonly found in spinach, but in concentrations approximately 25% lower than that of 20-hydroxyecdysone [11,31]. Polypodine B is less hydrophobic, and usually elutes slightly earlier, than 20-hydroxyecdysone [11]. We did not test for its presence. However, a small peak at 10.3 min elution time occurred on RP-HPLC traces obtained during the analysis of the temperature-induced upper and lower phases. This peak suggests the presence of polypodine B in our samples because it appeared only in the experimental samples and not the blanks. The peak was not verified with authentic compound.

4. Conclusions

Ecdysteroids could be extracted from spinach leaves with a solution of the copolymer UCON in water. UCON–Reppal–aqueous two-phase systems were successfully used to partition ecdysone and 20-hydroxyecdysone from the spinach plant. Additionally, purification of these ecdysteroids was made possible using temperature-induced phase separation. This ability supports previous research with authentic compounds of these ecdysteroids [30]. The technique is a relatively simple, rapid, inexpensive benchtop procedure that can be performed in a laboratory not equipped with sophisticated equipment and instrumentation (Fig. 2). An attractive advantage of temperature-induced phase separation is that slight chemical and/or physical manipulation increases selectivity and forces desired biocompounds into either water-rich or polymer-rich phases [32]. Ability to obtain ecdysteroids, destined for experimentation, in non-toxic media is desirable. However, phase-forming polymers are themselves non-toxic. An added benefit of this procedure is that it can easily be scaled-up for commercial use.

5. Acknowledgements

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