

Esterification of vertebrate-type steroids in the Eastern oyster (*Crassostrea virginica*)

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Abstract

Characteristics of acyl-coenzyme A (acyl-CoA):steroid acyltransferase from the digestive gland of the oyster *Crassostrea virginica* were determined by using estradiol (E2) and dehydroepiandrosterone (DHEA) as substrates. The apparent K_m and V_{max} values for esterification of E2 with the six fatty acid acyl-CoAs tested (C20:4, C18:2, C18:1, C16:1, C18:0, and C16:0) were in the range of 9–17 μM E2 and 35–74 pmol/min/mg protein, respectively. Kinetic parameters for esterification of DHEA (K_m : 45–120 μM ; V_{max} : 30–182 pmol/min/mg protein) showed a lower affinity of the enzyme for this steroid. Formation of endogenous fatty acid esters of steroids by microsomes of digestive gland and gonads incubated in the presence of ATP and CoA was assessed, and at least seven E2 fatty acid esters and five DHEA fatty acid esters were observed. Some peaks eluted at the same retention times as palmitoleoyl-, linoleoyl-, oleoyl/palmitoyl-, and stearoyl-E2; and palmitoleoyl-, oleoyl/palmitoyl-, and stearoyl-DHEA. The same endogenous esters, although in different proportions, were produced by gonadal microsomes. The kinetic parameters for both E2 (K_m : 10 μM ; V_{max} : 38 pmol/min/mg protein) and DHEA (K_m : 61 μM ; V_{max} : 60 pmol/min/mg protein) were similar to those obtained in the digestive gland. Kinetic parameters obtained are similar to those observed in mammals; thus, fatty acid esterification of sex steroids appears to be a well-conserved conjugation pathway during evolution. © 2004 Elsevier Inc. All rights reserved.

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1. Introduction

Vertebrate-like sex steroids, e.g. testosterone, androstenedione, and estradiol, have been found in several groups of invertebrates [1–5], including molluscs [6,7]. Although their origin is still a subject of controversy, a number of studies point to an endogenous source and a physiological role of these steroids in molluscs. Evidence in support of this possibility include the following: (i) several steroid biosynthetic pathways present in vertebrates have been identified [8–10]; (ii) a temporal variation in steroid titers and some biosynthetic pathways coinciding with reproductive stages have been found [8,11]; and (iii) alterations in sexual characteristics or reproduction have been observed when molluscs are exposed to estrogenic or androgenic compounds: e.g. 1-methyl-1,4-androstadiene-3,17-dione caused imposex (the imposition of male sexual characteristics in females) in

the whelk *Nassarius reticulatus* [12], and estradiol-induced vitellogenesis in the pacific oyster *Crassostrea gigas* [13].

Phase I metabolism of sex steroids, i.e. hydroxylation and reduction, has been detected in several protostome invertebrates. The presence of 20 α -, 17 β -, and 3 β -hydroxysteroid dehydrogenases have been demonstrated in crustacea, and C₁₇–C₂₀ lyase was found in the gonads of the crab *Cancer pagurus* [14,15]. Several monohydroxy-testosterone metabolites have also been detected in *Neomysis integer* and *Daphnia magna* [5,16], and 17 β -hydroxysteroids were demonstrated in *Homarus americanus* [17]. In mollusca, the presence of similar steroidogenic enzyme systems has been described. Steroid reductases and dehydrogenases were found in the gastropod *Clione antartica* [18], while 17 β -hydroxysteroid dehydrogenase, 5 α -reductase, 3 α -hydroxysteroid dehydrogenase, and an aromatization system were found in the snail *Helix aspersa* [6]. In bivalve molluscs, the presence of 3 β - and 17 β -hydroxysteroid dehydrogenases, C₁₇–C₂₀ lyase, and 5 α -reductase were also demonstrated in gonad homogenates of *Mytilus edulis* incubated with labeled precursors [8]. Aromatase activity

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was found in microsomal fractions isolated from the digestive gland of the mussel *Mytilus galloprovincialis* [10] and in gonad–digestive gland homogenates of the oyster *Crassostrea gigas* [9].

Despite the available information on Phase I metabolism of sex steroids in invertebrates, data regarding their conjugation are rather limited and based primarily on in vivo observations. For example, conjugated 5 α -dihydrotestosterone was detected in the ovary and the hemolymph of the decapod *Nephrops norvegicus*, although the nature of the conjugate was not described [19]. Glucuronyl and sulfate conjugates of progesterone metabolites (based on hydrolysis procedures) were observed in the gastropod *C. antartica* after exposure to [³H]progesterone [18]. Several studies have shown that other phenolic compounds are conjugated to polar metabolites with sulfate and glucosyl moieties in invertebrates [20–24], suggesting that polar conjugates of steroid hormones are also formed by sulfation and glucosidation.

Apart from this, apolar conjugation may also play a key role in invertebrates. Recently, apolar conjugation was shown to be the major pathway of testosterone metabolism (more than 70%) in the snail *Ilyanassa obsoleta* exposed to 1 μ M [¹⁴C]testosterone in water [25]. In addition, several invertebrates have been shown to readily convert sterols to fatty acid conjugates, although most of the knowledge is restricted to ecdysteroids [26–28]. Fatty acid esters of ecdysteroids are formed enzymatically in various insects species and are common metabolites in larvae and adults [29–31]. Also, gastropods have been shown to esterify sterols, such as cholesterol, brassicasterol, campesterol, stigmasterol, and β -sitosterol [32,33].

The present work was designed to assess the esterification (acyl-CoA:steroid acyltransferase activity) of two model steroids, estradiol and dehydroepiandrosterone, by microsomal fractions isolated from both the digestive gland and gonads of the Eastern oyster *Crassostrea virginica*. The digestive gland was selected because it is the tissue with a major metabolic role in bivalves, and gonads were chosen because they are considered to be the site for steroid synthesis. Estradiol and dehydroepiandrosterone were selected as model steroids because they are conjugated at different sites: DHEA is conjugated at the 3-OH position, and E2 is conjugated at the 17-OH position [34]. In addition, these steroids are normally used when assessing acyl-CoA:steroid acyltransferase in vertebrate species [35] and, therefore, facilitate inter-phyla comparisons.

2. Experimental

2.1. Chemicals

[2,4,6,7,16,17-³H]Estradiol (110 Ci/mmol) and [1,2,6,7-³H]dehydroepiandrosterone (60 Ci/mmol) were purchased from NEN Life Science Products Inc. Estradiol, dehydroepiandrosterone, and the lithium salt of acyl-CoA fatty acids

(oleoyl-CoA (C18:1), stearoyl-CoA (C18:0), arachinoyl-CoA (C20:4), palmitoyl-CoA (C16:0), linoleoyl-CoA (C18:2), and palmitoleoyl-CoA (C16:1)) were purchased from Sigma. All solvents were of HPLC grade and were purchased from Fisher Scientific.

2.2. Tissue preparation

Eastern oysters (*C. virginica*), 4 years old, were obtained from Prince Edward Island (Canada). They were maintained up to a month in a recirculating saltwater system and fed *Isochrysis galbana* and fish food pellets daily until dissection.

Digestive glands and gonads were dissected, frozen in liquid nitrogen, and kept at -80°C until used. Cellular fractions were prepared as described previously [36]. Samples were homogenized in ice-cold 10 mM Tris–HCl, pH 7.6, containing 150 mM KCl and 0.5 M sucrose, and centrifuged at $12,000 \times g$ for 45 min at 4°C . After centrifugation at $100,000 \times g$ for 90 min, pellets were washed and centrifuged for further 30 min. Finally, the pellet, termed microsomal fraction, was resuspended in 20 mM Tris–HCl, pH 7.6, containing 20% (w/v) glycerol.

Protein content was determined with the BCATM Protein Assay kit (Pierce Chemical Co.) according to the supplier's instructions using bovine serum albumin as a standard.

2.3. Enzyme assays

Assays for the esterification of estradiol and dehydroepiandrosterone were based on the method described by Xu et al. [37] with slight modifications. Briefly, microsomes (25–100 μ g protein) were incubated at 30°C for 30 min in a final volume of 0.25 ml 100 mM sodium acetate buffer, pH 6.0, in the presence of E2 (0.1–50 μ M; 0.05–2 μ Ci) or DHEA (0.1–150 μ M; 0.5–2 μ Ci) and 100 μ M of a fatty acid acyl-CoA. Endogenous conjugation was assayed in the presence of 1 mM CoA and 10 mM ATP. The reaction was stopped by the addition of 0.3 ml of ice-cold sodium acetate buffer, pH 6.0, and 4 ml ethyl acetate. The samples were vortexed immediately and centrifuged for 10 min at $3000 \times g$. The ethyl acetate extract was removed and evaporated to dryness under a stream of nitrogen. Each resulting residue was dissolved in 130 μ l of methanol and analyzed by HPLC.

Separation and measurement of the esterified metabolites were achieved by HPLC with a Spherisorb ODS column (5- μ m particle size, 250 mm \times 4.6 mm i.d.). The HPLC system consisted of a Waters 600E solvent gradient programmer, a Waters Lambda-Max model 481 UV detector, and a radioactive flow detector (β -ram from IN/US) as described by Xu et al. [37]. The solvent system consisted of acetonitrile/0.1% acetic acid in H₂O/methanol. The solvent gradient used for elution of the E2 esters from the column was: 12 min isocratic at 30/6/64; 6 min with a number 10 convex gradient to 60/0/40; 15 min isocratic at 60/0/40; 2 min with a number 2 convex gradient to 20/0/80; 5 min isocratic at

20/0/80; and the column was then returned to initial conditions over 15 min. The flow rate was 1.2 ml/min. DHEA esters were eluted in the isocratic mode with 100% methanol at the same flow rate.

3. Results

3.1. Optimization of the esterification assay

The activity of acyl-CoA:steroid acyltransferase in microsomal fractions isolated from the digestive glands of *C. virginica* was characterized using estradiol and oleoyl-CoA as substrates. The assay for E2 esterification was linear up to 0.1 mg microsomal protein/ml in the presence of 200 nM E2 (Fig. 1A), and at least up to 0.4 mg/ml in the presence of 25 μ M E2 (data not shown). Thereafter, all assays were performed using 0.1 mg microsomal protein/ml, except for endogenous esterification, which was studied using high protein concentrations to achieve high sensitivity (up to 1 mg/ml). The formation of oleoyl-E2 was measured as a function of the incubation time, and the reaction rate was linear for at least 40 min (Fig. 1B). All subsequent incubations were for 30 min. The reaction rate increased linearly with temperature over the range of 25–37 °C (Fig. 1C). An incubation temperature of 30 °C was selected for all further assays. Finally, the pH dependence of the enzymatic activity was studied, and its profile is shown in Fig. 1D. Acyl-CoA:steroid acyltransferase was active over a pH range

of 4.0–9.0 with an optimum around 6.0. Although optimization assays were performed at pH 5.0, which was the optimum for mammals, all subsequent assays for the molluscan enzyme were performed at pH 6.0.

In addition, microsomal protein was incubated in the absence of cofactors (CoA + ATP or fatty acid acyl-CoA), and the formation of E2 esters was also observed, although the activity measured was 20-fold lower than when a fatty acid acyl-CoA was added. When assays were performed using heat-inactivated microsomes (100 °C for 5 min), no esters were formed.

3.2. Esterification of estradiol in the digestive gland of *Crassostrea virginica*

E2 was esterified to the corresponding fatty acid ester when incubated with any of the six fatty acid acyl-CoAs used: arachidonoyl-CoA, palmitoleoyl-CoA, linoleoyl-CoA, oleoyl-CoA, palmitoyl-CoA, and stearoyl-CoA (Fig. 2). The retention time, and the apparent K_m and V_{max} for each of the different esters are shown in Table 1. K_m for all the esters ranged from 9 to 17 μ M; V_{max} differed over two-fold between esters and ranged from 35 to 74 pmol/min/mg protein.

Rates of conjugation were determined using saturating or near saturating amounts of E2 (25 μ M) and results are shown in Fig. 3. The rate of formation of linoleoyl-E2 conjugates was significantly lower (about two-fold difference, $P < 0.05$) than noted with the other esters, which occurred at similar rates.

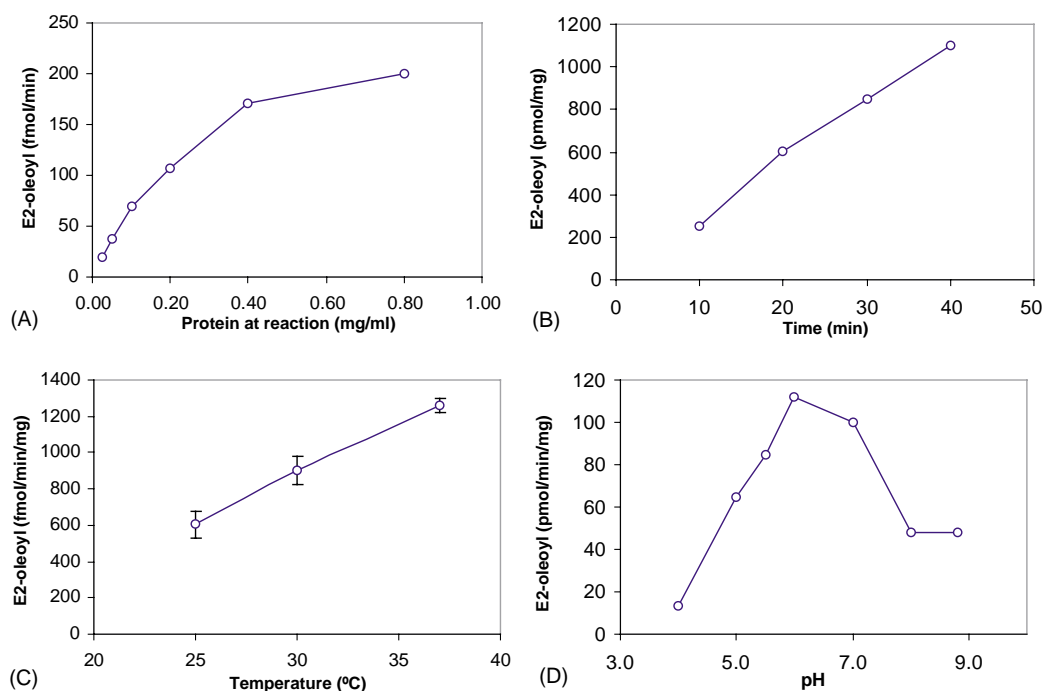


Fig. 1. Effect of the amount of microsomal protein in the reaction system (A), the incubation temperature (B), the incubation time (C), and the pH (D) on acyl-CoA:steroid acyltransferase activity using E2 and oleoyl-CoA as substrates. The concentration of E2 in the assay was 200 nM in experiments A and C and 25 μ M in experiments B and D.

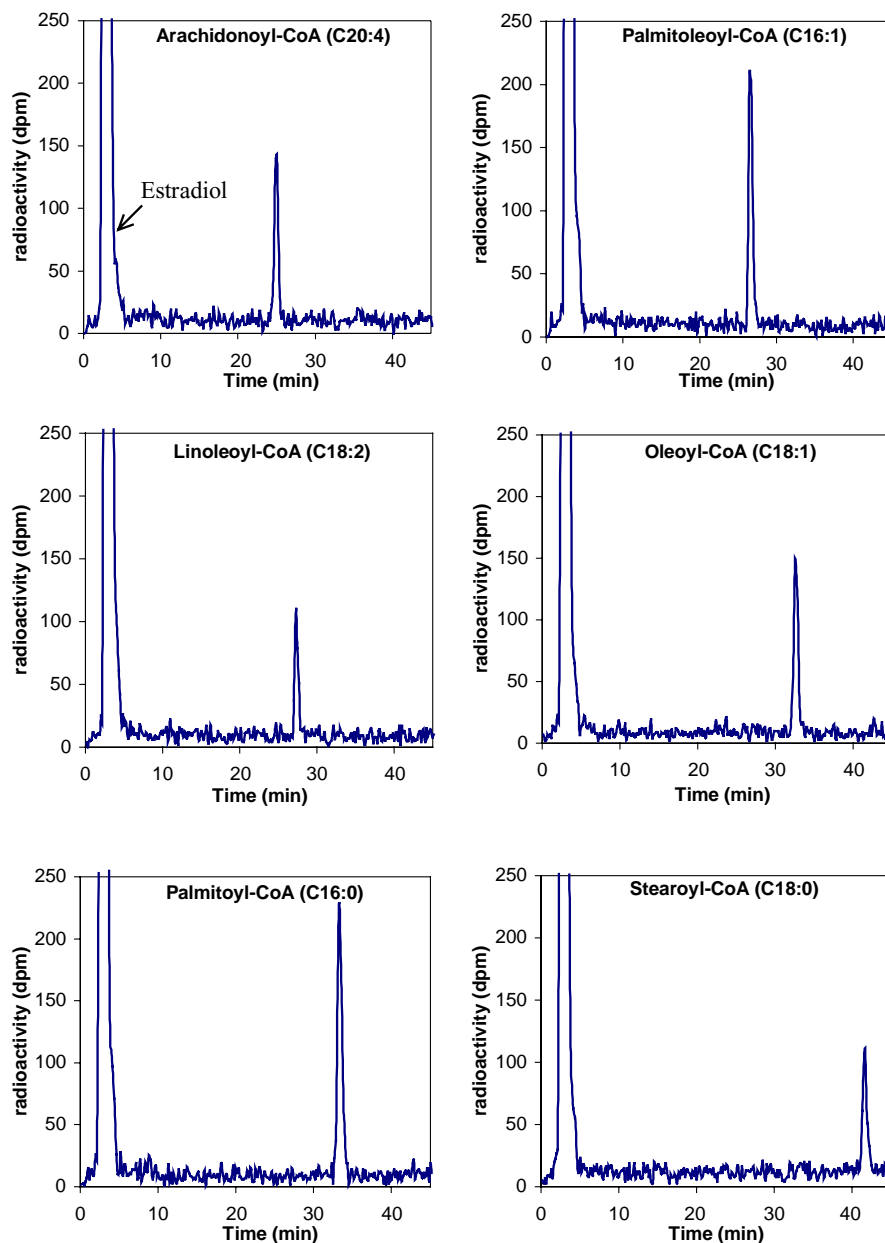


Fig. 2. Esterification of estradiol induced by the addition of specific fatty acid acyl-CoAs. In each experiment, 75 μ g of microsomal protein was incubated for 30 min with 25 μ M E2 at 30 °C.

Table 1

Retention times of E2 esters and kinetic parameters for esterification of E2 using different fatty acid acyl-CoAs as cofactors

Ester	Retention time	K_m	V_{max}
Arachidonoyl-E2	24:40	9.4 ± 1.8	55 ± 4
Palmitoleoyl-E2	26:33	11 ± 3	66 ± 8
Linoleoyl-E2	27:15	17 ± 5	35 ± 4
Oleoyl-E2	32:22	15.8 ± 1.6	53 ± 2
Palmitoyl-E2	33:12	10.1 ± 1.9	74 ± 6
Stearoyl-E2	41:34	12.3 ± 1.2	44.6 ± 1.7

Values (mean \pm S.D.) were obtained from reactions using five concentrations of E2 ranging from 3 to 50 μ M.

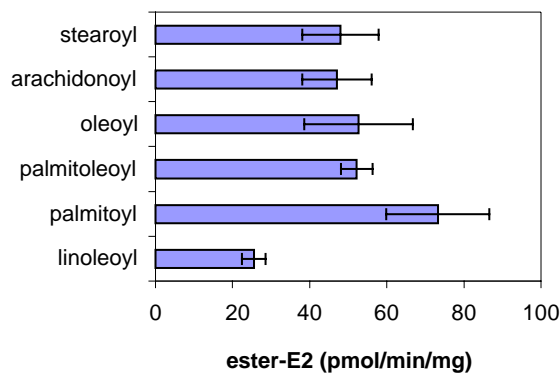


Fig. 3. Esterification of estradiol (25 μ M) with six different fatty acid acyl-CoAs. Values are the mean \pm S.E.M. ($n = 3$).

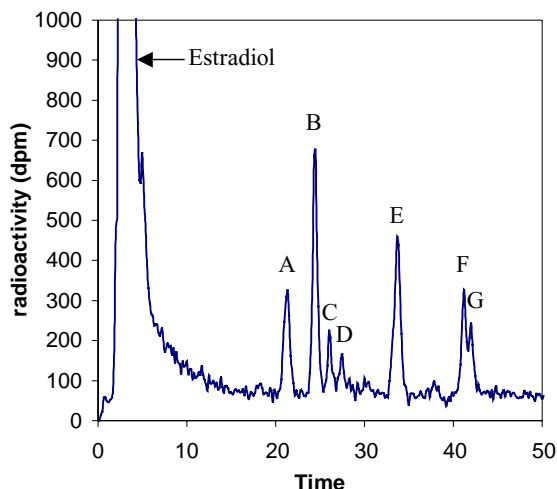


Fig. 4. Endogenous esterification of estradiol. 250 μg of digestive gland microsomal protein was incubated in the presence of 200 nM E2, 10 mM ATP, and 1 mM CoA for 30 min at 30 $^{\circ}\text{C}$. A and B: unknown; C: palmitoleyl-E2; D: linoleoyl-E2; E: oleoyl/palmitoyl-E2; F + G: stearoyl-E2.

Endogenous esterification of E2 assessed by incubation of microsomal fractions in the presence of ATP and CoA resulted in the formation of up to seven peaks (Fig. 4). Peaks C, D, E, and F + G were tentatively identified as palmitoleyl-E2, linoleoyl-E2, oleoyl/palmitoyl-E2, and stearoyl-E2, respectively, by comparison of their retention times with those obtained in the presence of specific fatty acid acyl-CoA standards (Table 1). Peaks A and B are unknown. No qualitative differences were observed in the chromatogram profiles when microsomes were incubated in the presence of low (200 nM) and high (25 μM) E2 concentrations. Esterification rates increased linearly with E2 concentration in the range of 200 nM–25 μM .

3.3. Esterification of dehydroepiandrosterone in the digestive gland of *Crassostrea virginica*

Similarly to E2, DHEA was esterified to the corresponding fatty acid ester when incubated with any of the six fatty

Table 2
Retention times of DHEA esters and kinetic parameters for esterification of DHEA using different fatty acid acyl-CoAs as cofactors

Ester	Retention time	K_m	V_{max}
Arachidonoyl-DHEA	11:51	109 ± 27	52 ± 8
Palmitoleyl-DHEA	13:14	120 ± 16	182 ± 14
Linoleoyl-DHEA	14:15	79 ± 17	51 ± 5
Oleoyl-DHEA	18:02	45 ± 10	30 ± 4
Palmitoyl-DHEA	18:29	54 ± 6	81 ± 4
Stearoyl-DHEA	26:38	60 ± 8	87 ± 5

Values (mean \pm S.D.) were obtained from reactions using five concentrations of DHEA ranging from 3 to 150 μM .

acid acyl-CoA substrates studied. The apparent K_m and V_{max} for each of the different esters were calculated, and results are shown in Table 2. K_m values ranged from 45 to 120 μM , and V_{max} values ranged from 30 to 182 pmol/min/mg protein. At a DHEA concentration of 25 μM , the esterification rate was lower than that of E2 for all the esters tested (2.7 ± 0.5 -fold).

Up to five peaks were observed when microsomal protein was incubated with DHEA in the presence of ATP and CoA (Fig. 5). Peaks C, D, and E were tentatively identified as palmitoleyl-DHEA, oleoyl/palmitoyl-DHEA, and stearoyl-DHEA, respectively, whereas peaks A and B are unknown.

3.4. Esterification of estradiol and dehydroepiandrosterone in gonads of *Crassostrea virginica*

Gonadal microsomes incubated in the presence of ATP and CoA formed the same number of peaks at similar retention times as those detected previously in the digestive gland microsomes. However, the profile of the esters was different (Fig. 5).

When the esterification of E2 was assayed in the presence of palmitoyl-CoA, we found a K_m of $9.7 \pm 0.9 \mu\text{M}$ and a V_{max} of 38 ± 2 pmol/min/mg protein. This K_m was similar to that found in the digestive gland (Table 1). In contrast, when esterification of DHEA was assayed in the presence of palmitoleyl-CoA, the K_m was $61 \pm 22 \mu\text{M}$ and the V_{max}

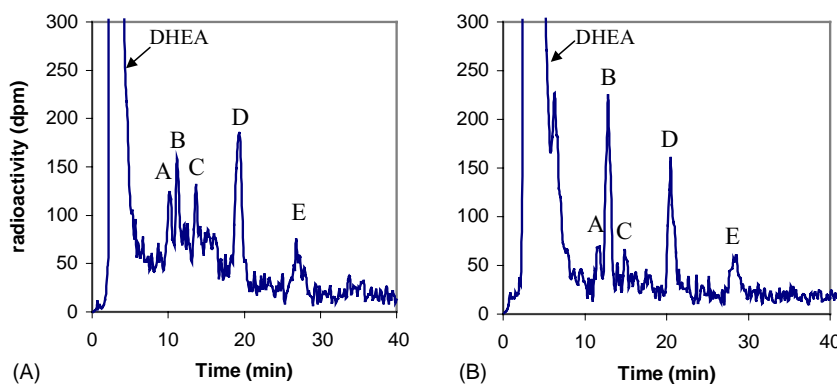


Fig. 5. Endogenous esterification of dehydroepiandrosterone by digestive gland (A) and gonad (B) microsomes. 80 μg of microsomal protein was incubated in the presence of 1 μM DHEA, 10 mM ATP, and 1 mM CoA for 30 min at 30 $^{\circ}\text{C}$. A and B: unknown; C: palmitoleyl-DHEA; D: oleoyl/palmitoyl-DHEA; E: stearoyl-DHEA.

was 60 ± 10 pmol/min/mg protein. This K_m was lower than that found in the digestive gland for the same conjugate, although it is in the range of other DHEA esters (Table 2). The V_{max} values for the two conjugative reactions assayed in the gonads were lower than those found in the digestive gland.

4. Discussion

In the present study, we characterized the acyl-CoA:steroid acyltransferase activity in the microsomal fraction of digestive glands and gonads from the oyster *C. virginica*. Activity increased linearly with time (10–40 min), amount of protein (25–100 μ g/ml), and temperature (25–37 °C), and was active over a broad range of pH values. The profile of the pH dependence of the activity was very similar to that found for the fatty acyl-CoA:ecdysteroid-22-*O*-acyltransferase in the tobacco budworm *Heliothis virescens* [26], with very low activities at pH values less than 4 and considerable activities at basic pH values. The optimum pH, 6.0, was also in the same range of those reported earlier for the fatty acyl-CoA:ecdysteroid acyltransferase in *H. virescens* and the fatty acyl-CoA:estradiol acyltransferase in rats [26,37].

Oyster tissue microsomes isolated from digestive glands and gonads esterified E2 and DHEA with all the fatty acid acyl-CoA substrates tested, which included totally saturated fatty acids (C18:0 and C16:0), monounsaturated fatty acids (C18:1 and C16:1), and polyunsaturated fatty acids (C18:2 and C20:4). Several steroid fatty acid esters (at least seven E2 esters and five DHEA esters) were formed when digestive gland or gonad microsomal fractions were incubated with ATP and CoA. According to their retention times, the esters formed were putatively palmitoleoyl-, stearoyl-, and oleoyl/palmitoyl-DHEA as well as palmitoleoyl-, linoeloyl-, oleoyl/palmitoyl-, and stearoyl-E2. This indicates the presence of similar enzymatic systems in both tissues. The fatty acid esters of steroids found in this study have been reported to be major fatty acid esters in several molluscan species [38–40]. Namely, palmitoyl, stearoyl, palmitoleoyl, and oleoyl represented 17.9, 6.2, 7.2, and 9.1%, respectively, of the total fatty acids detected in the bivalve *M. galloprovincialis* [40]. Some other esters (peaks A and B in Figs. 4 and 5) were formed in the presence of ATP and CoA (endogenous esterification), but they did not coelute with any of the six esters analyzed in this study. Their retention times suggest that they might be polyunsaturated esters.

We demonstrated the ability of preparations from the digestive gland and gonads of the Eastern oyster to esterify both E2 and DHEA. Since DHEA can only be esterified at the 3 β -OH position, and assuming that, similar to mammals, E2 is esterified at the 17 β -OH position [34], our results would indicate that the oyster can esterify sex steroids at both the 17 β -OH and the 3 β -OH positions. The K_m values for DHEA were higher than those for E2. In mammals, there is still controversy with respect to whether there are specific enzymes for the esterification of 3-OH and 17-OH

positions or not. DHEA has been shown to act as a competitive inhibitor of E2 esterification, suggesting that the same enzyme esterifies both steroids [41]. A similar fatty acid composition for both steroid esters has also been reported supporting the hypothesis of a single enzyme isoform [35]. In general, the HPLC chromatograms we obtained for endogenous conjugation (Figs. 4 and 5) also showed a similar fatty acid composition for both steroid esters and comparable rates of conjugation between different CoA esters for both steroids.

Apolar metabolism of steroids is known to occur in molluscs. De Souza and De Oliveira [32] reported a 15.4% esterification of [¹⁴C]cholesterol when incubated in the hemolymph of the mollusc *Biomphalaria glabrata* in the absence of cofactors for 24 h at 37 °C. Several studies suggest that the esterification of cholesterol and other steroids occurs via separate forms of acyl-CoA acyltransferase. In mammals, the profiles of the cholesterol esters formed differ from those of the estradiol esters. Moreover, a potent acyl-CoA:cholesterol acyltransferase inhibitor had no effect on the esterification of E2 or DHEA [34,42].

In contrast to the limited information on the esterification of sex steroid metabolism in molluscs and other invertebrates, there exists an extensive literature on esterification of ecdysteroids in several arthropod species [26,27,43–45], which might provide some insight on a possible role for other steroid esters. In the cricket *Acheta domesticus* [44] and the cockroach *Periplaneta americana* [45], the esters are synthesized in the ovary or transferred to it and then to the eggs, where they represent a storage form of the molting hormone [43] that supplies free steroid during embryogenesis [46]. In fact, the storage role of steroid esters is not restricted to insects. Steroid esters have been used pharmacologically for decades as potent long-acting pharmaceuticals [47] that require enzymatic hydrolysis by esterases to exert their endocrine actions [48].

From a comparative approach, apolar conjugation of steroids in the oyster *C. virginica* saturates at similar concentrations (viz. K_m was 13 μ M versus 8 μ M E2 for E2 oleoate formation in the oyster digestive gland and rat liver, respectively) and occurs at a similar rate to that found in mammals. At 25 μ M E2, 53 and 40 pmol oleoyl-E2/min/mg protein were formed by oyster digestive gland and rat liver, respectively (Mesia-Vela et al., unpublished data). These kinetic parameters were also very similar to those reported for acyl-CoA:ecdysteroid-22-*O*-acyltransferase (K_m : 10 μ M; V_{max} : 85 pmol/min/mg) in midgut tissues of the tobacco budworm *H. virescens* [26].

To our knowledge, no physiological differences related to the nature of the fatty acid moiety of a steroid ester are known. If the affinity of steroid esterase(s) differed for different esters, the fatty acid composition of the steroid esters could have physiological relevance. The available information on steroid esterases is rather restricted to mammals. A study using the MCF-7 breast cancer cell line showed that long chain esters, such as the ones studied here, are

hydrolyzed by specific esterases distinct from those that act upon shorter chain esters of E2 (e.g. acetate, propionate, etc.), which are nonspecific esterases [48]. Certainly, further work is needed to elucidate whether invertebrate esterases act upon long or short chain esters and to determine the affinities of these enzymes for the various esters.

Both acyl-CoA acyltransferases and esterases can be modulated by drugs [37]. Compounds that alter esterification or ester cleavage will subsequently modulate hormone availability and activity and may well act as endocrine disrupting substances. In this sense, the effect of different concentrations of TBT on the activity of fatty acid acyl-CoA:steroid acyltransferase was explored in preliminary studies in our laboratory. Preliminary data showed that TBT in the low micromolar range inhibited esterification of E2 and DHEA in vitro, with the esterification of E2 being more sensitive. TBT is an antifouling agent that causes imposex in some gastropod species. Its ability to interact with different steroid metabolic pathways, aromatase, and 5 α -reductase, has already been shown [49–51]. However, interaction with conjugation enzymes has been hypothesized as a more plausible mode of action to explain the increased testosterone levels detected in some TBT exposed molluscs [25,52]. The interference of TBT in the esterification of free hormones can affect levels of active steroids within tissues and may be one of the responsible mechanisms of the reported androgenization/feminization phenomena. In fact, esterification of testosterone has already been considered as a possible site of action for the endocrine disrupter TBT [25,53], although further experiments to test this hypothesis are warranted.

In summary, data obtained on *C. virginica* shared many similarities with data available for vertebrate and ecdysteroid esterification in insects, suggesting that esterification is a highly conserved conjugation pathway in evolution. Together with other biosynthesis and conjugation pathways, esterification could modulate endogenous steroids levels in molluscs. Clearly, further research to gain better understanding of the physiological function of the esterification of steroids in marine organisms is needed.

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