J.Biosci., Vol. 1, Number 2, June 1979, pp. 159-168. © Printed in India.

Alpha-tocopheryl acetate hydrolase in chicken liver. Characterisation and properties

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MS received 24 July 1978; revised 10 November 1978

Abstract. An enzyme catalysing the hydrolysis of *a*-tocopheryl acetate was characterised in chicken liver. The enzyme was localised in the microsomes, had an optimum pH 8·6 and a K_m value of 0·5 mM. The enzyme did not hydrolyse retinyl acetate, cholesteryl acetate and ethyl acetate, thus indicating a high degree of specificity. *a*-Tocopheryl acetate hydrolase required bile salts as a specific co-factor. The results suggested a role for this enzyme in the absorption of vitamin E.

Keywords. Alpha-tocopheryl acetate hydrolase; chicken liver; bile salts.

Introduction

The presence of an enzyme in animal tissues hydrolysing α-tocopheryl esters was indicated by the observation that tocopheryl acetate was absorbed partly in the free form (Weiss *et al.*, 1964). We have confirmed the presence of such an enzyme hydrolysing α-tocopheryl acetate in the liver and pancreas of chicken (Bai *et al.*, 1972). Extensive literature is available on ali-esterase (Burch, 1954), cholesteryl acetate hydrolase (Korzenovsky *et al.*, 1960) and retinyl acetate hydrolase (Ganguly, 1967). In view of the presence of these enzymes in the liver, a systematic examination of the properties like the cofactor requirements and specificity of the hepatic α-tocopheryl acetate hydrolase in chicken was undertaken in the present study.

Materials and methods

Materials

Vitamin A acetate was obtained from Hoffman-La-Roche, Basle, Switzerland. Cholesteryl acetate, ethyl acetate, sodium taurocholate and $\alpha\alpha'$-dipyridyl were obtained from BDH, Poole, England. α-Tocopheryl acetate, quinine sulphate and ferric chloride were the products of E, Merck, Dermstadt, Germany. Eserine sulphate was from Boehringer, Ingelheimam Rhein, Germany, and *p*-chloro-mercuribenzoate from Fluka AG, Buchs SG, Switzerland. N-Ethylmaleimide and

reduced glutathione were from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals were of the analytical reagent grade. Chicken of both sexes of about 0·5 to 0·8 kg body weight were obtained from the local market.

Methods

Preparation of the substrates. Substrate dispersions were made using small amounts of ethanol (0·05 ml) as dispersing agent as described by Mahadevan *et al.* (1961). Other emulsifiers when used were dispersed in appropriate buffers and added.

Preparation of the homogenate. Initial experiments were done using 10% w/v chicken liver homogenate in 0·25 M sucrose. The homogenate (10% w/v) in 0·25 M sucrose containing 0·02 M Tris HCl buffer, pH 7·6 and 0·1 mM EDTA gave higher α-tocopheryl acetate hydrolase activity and was used for later experiments. Subcellular fractionation was carried out following the procedure of Schneider and Hogeboom (1950). The post-mitochondrial fraction was found to contain the bulk of the enzyme activity and this has been employed as the enzyme source in routine assay.

Enzyme assays. Cholesterol acetate hydrolase (E.C. 3.1.1.13), retinyl acetate hydrolase (E.C. 3.1.1.12) and ali-esterase (E.C. 3.1.1.1) were assayed as described by Krishnamurthy *et al.* (1958). α-Tocopheryl acetate hydrolase activity was determined essentially as described earlier (Bai *et al.*, 1972) except that NN′ diphenyl-*p*-phenylene diamine was omitted from the incubation medium. One ml of 3 % sodium taurocholate in veronal buffer, pH 8.6 was included in a total volume of 5 ml reaction mixture unless otherwise specified.

Enzyme activities are expressed as *μg* of liberated alcohols when retinyl acetate, cholesterol acetate and α-tocopheryl acetate were used and for the ali-esterase, amount of 0·01 N NaOH required to neutralise the liberated acid under the conditions of assay.

Protein was estimated by the method of Lowry *et al.* (1951).

Paper chromatography. The reaction of α-tocopheryl acetate hydrolase was terminated by adding an equal volume of ethanol. The supernatant solution was extracted with petroleum ether, evaporated under reduced pressure and dissolved in a small volume of petroleum ether. This was subjected to uni-dimensional ascending paper chromatography using paraffin coated Whatmann No. 1 filter paper, with 80% ethanol, as the solvent.

Results

Characterisation of the reaction products

In addition to α-tocopherol, its quinones can also give a positive colour in the Emmerie-Engel reaction, it was necessary to identify the product of the reaction. The product of the α-tocopheryl acetate hydrolase reaction gave an R_1 value of 0·82, same as that of standard α-tocopherol. Further, on co-chromatography with α-tocopherol the product gave a single spot of R_1 value 0·82, thereby confirming the identity of the product as α-tocopherol.

Properties of the enzyme reaction

The optimum pH for the tocopheryl acetate hydrolysis was 8·6. The amount of α-tocopherol produced increased linearly with the amount of protein in the reaction mixture upto 30 mg. The activity was linear upto 30 min when 20 mg protein was used. The K_m value of 0·5 mM was calculated from the double reciprocal plot.

Krishnamurthy *et al.* (1958) reported that in chicken liver, the nuclear fraction had more cholesteryl acetate hydrolase than the microsomal fraction; while the retinyl acetate hydrolase activity was more closely distributed in the two fractions, although the microsomes accounted for about 60% of the activity of the whole homogenate. The distribution pattern for the α-tocopheryl acetate hydrolase as given in table 1, however, showed that the activity seemed to be localised in the microsomal fraction, with the other cellular fractions showing less than 10% of activity. It may be noted that the activity of the whole original homogenate was more than the algebraic sum of the individual fractions. However, by reconstituting the homogenate by adding the individual fractions, the original activity was recovered.

Storage and heat treatment

Post-mitochondrial supernatant of chicken liver homogenate was kept at 4° C and aliquots analysed for the four hydrolases on different days. The activities towards the hydrolysis of vitamin A and E acetates were more labile than the other two (figure 1). α-Tocopheryl acetate hydrolase upto 6 days, retinyl acetate hydrolase for 14 days and the other two hydrolases for about three weeks, retained their activities on storage. Figure 2 shows the effect of heat treatment on the four hydrolases. α-Tocopheryl acetate hydrolase was the most resistant to heat denaturation since even at 65° C, its activity was fully retained, while the other three activities were almost completely abolished at this temperature.

Effect of metallic ions, sulphydryl inhibitors and alkaloids

The effect of metal ions at 6 mM, the concentration employed by Sastry *et al.* (1957), on the four activities are summarised in table 2. Activation by Ca^{2+} (3-fold) and Mg^{2+} (2-fold) was a unique feature of α-tocopheryl acetate hydrolase because these ions were without effect on the other three enzymes. The other metal ions had similar effects on all the four enzymes. Eserine sulphate at higher concentrations inhibited all the four activities, while quinine sulphate inhibited only the cholesterol acetate hydrolase (table 3). Among the sulphydryl reagents, *p*-hydroxymercuribenzoate markedly inhibited all the three activities excepting the hydrolysis of vitamin A acetate. However, reduced glutathione (GSH) added in equimolar concentrations could markedly reverse the *p*-hydroxymercuribenzoate inhibition of all the three activities. With N-ethyl maleimide, a lower degree of inhibition was seen only with α-tocopheryl acetate hydrolase and cholesterol acetate hydrolase and not with the other two activities.

Effect of emulsifying agents

Sodium taurocholate and deoxycholate as well as synthetic detergents were added to the reaction mixture to ascertain the effect of these compounds on the

Table 1. Intracellular distribution of α-tocopheryl acetate hydrolase in chicken liver.

Source	α-tocopherol liberated (μg)	Percentage activity
Whole homogenate	430	100
Nuclear fraction	43	10
Mitochondrial fraction	28	6
Microsomal fraction	258	60
Supernatant fraction	34	8
Nuclear + mitochondrial + microsomal + supernatant	421	98

The assay mixture consisted of 2600 μg α-tocopheryl acetate, 30 mg sodium taurocholate, 20 mg enzyme-protein and veronal buffer, 0·05 M, pH 8·6, in a final volume of 5 ml. Incubation was for 30 min at 37° C. At 0 and 30 min, 1 ml aliquots were withdrawn, added to 1 ml ethanol and extracted with petroleum ether. α-Tocopherol liberated was estimated by the Emmerie-Engel reaction.

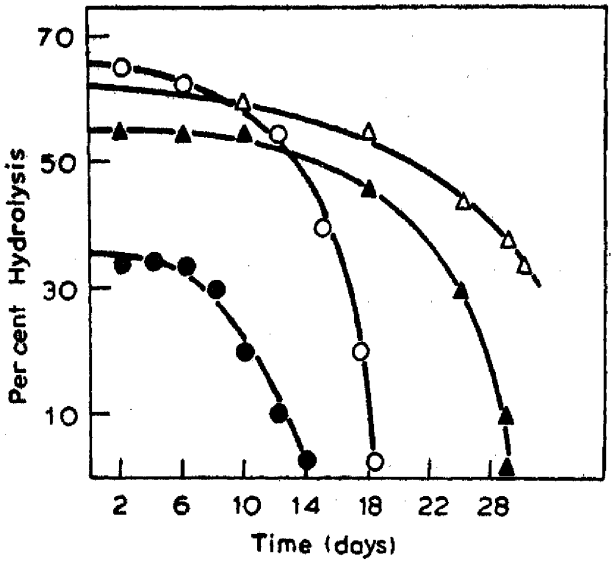

Figure 1. Effect of storage on the four enzyme activities. Aliquots were assayed for the four activities on different days as described in the text.

o Retinol acetate hydrolase
● *a*-tocopheryl acetate hydrolase
▲ Cholesteryl acetate hydrolase
Δ Ali-esterase

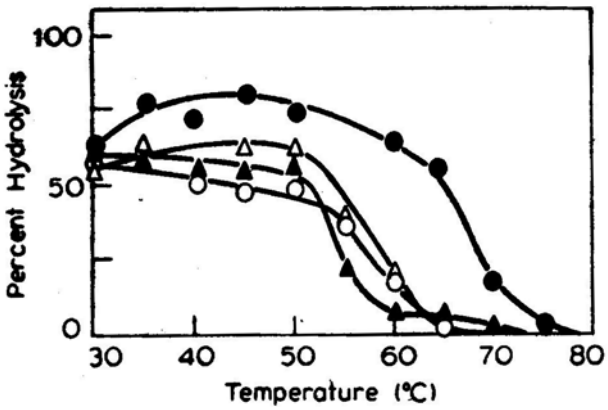

Figure 2. Heat denaturation of the four enzyme proteins. Enzymes were kept at required temperature for 10 min (in a water bath), chilled and immediately assayed for the four activities as given in the text.

o Retinol acetate hydrolase
● *α*-tocopheryl acetate hydrolase
▲ Cholesteryl acetate hydrolase
Δ Ali-esterase

activity of the four hydrolases (table 4). It can be seen from the table that sodium taurocholate enhances the activity of only *α*-tocopheryl acetate and cholesterol ester hydrolases. The synthetic detergents, while increasing the activity of cholesterol ester hydrolase, had no effect on the activity of *α*-tocopheryl acetate hydrolase. Further, they inhibited the enhanced activity of *α*-tocopheryl acetate hydrolase in the presence of sodium taurocholate.

Table 2. Effect of metal ions on the four ester hydrolases of chicken liver.

Additions	Concentration (mM)	Percentage activity			
		α-Tocopheryl acetate hydrolase	Retinyl acetate hydrolase	Cholesteryl acetate hydrolase	Ali-esterase
Nil		100	100	100	100
$CuSO_4$	6	2	5	27	8
$CaSO_4$	6	300	105	72	63
	0·1	135	100	78	81
$MgCl_2$	6	200	60	66	102
	0·1	125	83	72	110
$CdCl_2$	6	3	13	25	15
$HgCl_2$	6	8	10	38	2
$ZnSO_4$	6	0	8	30	4

Experimental details were as given in table 1 except that metal ions at concentrations indicated were included in the reaction mixture. Activities in the absence of any metal ions were given as 100

Table 3. Effect of sulphydryl compounds and alkaloidal reagents on the four ester hydrolases from chicken liver.

Addition	Concentration (mM)	Percentage inhibition			
		Tocopheryl acetate hydrolase	Retinyl acetate hydrolase	Cholesterol acetate hydrolase	Ali-esterase
Eserine sulphate	6	100	90	85	100
	0·6	100	58	40	80
N-ethyl maleimide	6	50	0	60	5
	0·6	27	0	10	8
P-Hydroxy mercuribenzoate	6	40	0	85	100
	0·6	40	0	15	12
Quinine sulphate	6	5	0	50	0
	0·6	12	0	30	0

Details were as given in table 2. Activity in the absence of any added compound was taken as 100 and activity in the presence of added compound is taken as percentage of this. Additives were prepared in buffer.

Table 4. Effect of emulsifying agents on the activity of the four esterases of chicken liver.

Addition	Amount (mg)	Alcohol liberated (μg)			0·01 N NaOH (ali-esterase units)
		α-tocopherol	retinol	cholesterol	
None	—	0	810	140	4·0
Sodium taurocholate	5	100	900	1000	3·5
,,	10	300	945	1440	3·3
,,	30	450	950	1720	3·0
,,	50	400	880	1180	2·8
Sodium deoxycholate	30	425	915	1400	3·0
Teepol	30	0	5	10	0·0
Gum acacia	30	0	870	40	3·8
Tween–80	30	0	890	1320	3·6
Tween–20	30	0	1170	1480	3·1
Lissapol–N	30	0	1020	1620	3·5
Triton X–100	30	0	1125	1220	3·8
Triton X–100 + sodium taurocholate	30 30	315	945	1600	3·8
Tween–80 + sodium taurocholate	30 30	278	975	1640	3·8

The reaction mixture for α-tocopherol, retinol or cholesterol acetate hydrolase contained 2500 μg of α-tocopheryl acetate, retinyl acetate or cholesteryl acetate, 30 mg of sodium taurocholate and 20 mg of protein. Veronal buffer pH 8·6 was used for α-tocopheryl acetate and retinyl acetate hydrolases and phosphate buffer 0·05M, pH 7·0 was employed for cholesterol hydrolase. Incubation was at 37° C for 30, 60 and 120 min for the three hydrolases, respectively. Ali-esterase was determined in an incubation system containing 2·5 ml of 5% ethyl acetate, 20 mg protein, 75 mg sodium taurocholate and veronal HCl buffer, 0·05 M, pH 8·6 in a final volme of 12·5. ml. Five ml aliquots were used for titration against 0·01 N NaOH. Specified amount of emulsifier was added as aqueous suspension.

Effect of pre-treatment with sodium taurocholate and GSH

Hyun *et al.* (1969) have shown that rat pancreatic cholesterol ester hydrolase required taurocholate for the activity and also that taurocholate protected the enzyme against tryptic inactivation. The addition of bile salts to the *p*-hydroxymercuribenzoate-inhibited enzyme could not reverse the inhibition. However, the taurocholate pre-treated enzyme was not inhibited by *p*-hydroxymercuribenzoate. In the present study, the pre-treatment of the liver homogenate with 1 mM GSH resulted in no appreciable change of all the four activities (table 5). Since taurocholate was found to be essential for α-tocopheryl acetate hydrolase, an aliquot (1 ml) of the liver preparation was treated with 30 mg of sodium taurocholate for 30 min at 4° C, the samples were then assayed for activity. α-Tocopheryl acetate hydrolase was inhibited to about 50% while cholesteryl acetate hydrolase was slightly activated, and no effect was noticed for the other two activities as compared to the untreated samples (table 4). On addition of *p*-hydroxymercuribenzoate to the sodium taurocholate-pre-treated enzymes, inhibition was noticed for cholesteryl acetate hydrolase, α-tocopheryl acetate hydrolase and ali-esterase. But simultaneous addition of *p*-hydroxymercuribenzoate (1 mM) and GSH (1 mM) to the taurocholate-pre-treated sample, showed that inhibition persisted in the case of only α-tocopheryl acetate hydrolase, although GSH could reverse the inhibition for cholesteryl acetate hydrolase and ali-esterase. It may be seen from the results in tables 3 and 5 that when GSH- and *p*-hydroxymercuribenzoate were simultaneously supplied to the reaction system, the inhibition of α-tocopheryl acetate hydrolase could be partly reversed (83 to 25%).

Table 5. Effect of *p*-hydroxymercuribenzoate on the enzyme pre-treated with GSH or sodium taurocholate.

Treatment	Percentage activity			
	Tocopheryl acetate hydrolase	Retinyl acetate hydrolase	Cholesteryl acetate hydrolase	Ali-esterase
Enzyme pre-treated with GSH (1 mM)	100	100	100	100
Enzyme pre-treated with sodium taurocholate (30 mg)	52	97	110	88
Enzyme pre-treated with sodium taurocholate + *p*-hydroxymercuribenzoate	8	89	6	20
Enzyme pre-treated with sodium taurocholate + *p*-hydroxymercuribenzoate (1 mM) + GSH (1 mM)	11	93	97	98

The enzyme was pre-incubated with glutathione (1 mM) or sodium taurocholate (30 mg/20 mg protein) at 4° C for 30 min at the end of which aliquots were assayed for the hydrolase activities. Other details are as given in table 1.

Attempts at solubilisation

α-Tocopheryl acetate hydrolase could not be solubilised from the acetone powder of chicken liver using sucrose (0·88 M, 0·45 M, 0·25 M), phosphate, tris or veronal buffers of varying pH values or saline of different molarity. However, extraction of the acetone powder with phosphate buffer resulted in the solubilisation of the ali-esterase. Addition of detergents to the extraction buffers resulted either in the extraction of the remaining three enzymes together or inactivation of *α*-tocopheryl acetate hydrolase. For example, Lissapol-N treatment of acetone powder inhibited the *α*-tocopheryl acetate hydrolase activity while the other three activities were solubilised to some extent. In view of the failure to solubilise the enzyme, it was not possible to purify the enzyme.

Discussion

The results of the present study would suggest the presence of an enzyme hydrolysing *a*-tocopheryl acetate in chicken liver and confirms our earlier preliminary report (Bai *et al.*, 1972). In the present report, a detailed investigation of the properties of *a*-tocopheryl acetate hydrolase has been carried out and comparison made with that of the other three well-recognised hydrolases (cholesterol, retinol and ali-esterase). *a*-Tocopheryl acetate hydrolase showed only a single pH optimum of 8·6 while retinol acetate hydrolase, cholesterol acetate hydrolase and ali-esterase have been reported to have a different pH profile (Ganguly, 1967). The intracellular distribution of the *a*-tocopheryl acetate hydrolase was not the same as for the other three hydrolases; the bulk of the activity was associated with the microsomal fraction and a smaller amount in the nuclear fraction.

The requirement for the specific emulsifier, sodium taurocholate or deoxycholate in the assay system for the demonstration of the α-tocopheryl acetate hydrolase activity supports the view that this enzyme is distinct from the other three enzymes. It is seen that cholesterol acetate hydrolase also required the presence of an emulsifier for its activity, but the requirement was non-specific, since all the detergents studied were equally potent (table 4). These results could be taken as suggestive of a role other than as a mere emulsifying agent for the bile salts, probably a specific co-factor function. Bile salts have been shown to be essential for rat liver cholesterol esterase (Ganguly, 1967). David *et al.* (1966) have attributed a co-factor function for bile salts in the case of rat liver cholesterol esterase while Hyun *et al.* (1969) have shown an obligatory requirement of bile salts for rat pancreatic cholesterol esterase. However, our results do not show any such specific requirement for bile salts for the activity of hepatic cholesterol esterase in the case of chicken.

A similar requirement for trihydroxy bile salts has been reported by Hyun *et al.* (1969) in the case of rat pancreatic juice cholesterol esterase. These authors have speculated that the bile salts may form a complex with hydrogen bonding between the hydroxyl group and allosteric site of the protein leading to conformational change of the enzyme protein.

Tocopheryl acetate hydrolytic activity was profoundly enhanced by the presence of Ca^{2+} or Mg^{2+} in the reaction mixture which is a distinct feature of this enzyme, since these ions had no effect on the other three activities. Storage and heat

inactivation studies provide additional evidence for the presence of a specific *a*-tocopheryl acetate hydrolase since it was the only activity retained at 65° C at which temperature all the other three activities were almost completely abolished. Preferential solubilisation of chicken liver retinol and cholesterol acetate hydrolases and ali-esterase with Lissapol-N (Ganguly, 1967) suggests that *a*-tocopheryl acetate hydrolase is an enzyme distinct from other hydrolases.

The physiological significance of this enzyme present in the tissue was reported in our earlier communication (Bai *et al.*, 1972). Vitamin E absorption is also believed to be through the lymphatic route and the presence of an enzyme has been suggested by the earlier studies of Weiss *et al.* (1964) who have shown that *a*-tocopheryl acetate is absorbed partly as ester and partly as free *a*-tocopherol. Gallo-Torris (1970) has confirmed that in rat, with bile duct cannula, *a*-tocopheryl acetate is absorbed partly after hydrolysis in the intestine and partly as unchanged ester into the lymph. Krishnamurthy and Bieri (1963) in an earlier report have shown that the absorption of *a*-tocopherol is incomplete in rats and chicken and this has been confirmed in man (Kelleher and Losowsky, 1970). Tocopheryl acetate hydrolase activity was localised only in the liver and pancreas (Bai *et al.,* 1972) while vitamin A esterase and cholesterol esterase are present in small intestine also (Ganguly, 1967). This would probably explain why vitamin A absorption is more efficient than vitamin E absorption, since the hydrolysis of the E-ester is probably mediated by the enzyme secreted from the pancreas into the intestine while vitamin A ester hydrolysis is achieved by the intestinal enzyme itself. Another factor that might influence the rate of vitamin E absorption is the obligatory requirement of bile salts for the *a*-tocopheryl acetate hydrolase activity (as shown in the present studies) while retinyl acetate hydrolase does not show any specific requirement for bile salts for its activity. The essentiality of bile salts for the optimum absorption of vitamin E acetate has been confirmed by Gallo-Torris (1970) who has demonstrated that in the absence of bile, labelled vitamin E acetate is absorbed in the esterified form itself without undergoing hydrolysis and that too in much reduced amounts. Muller *et al.* (1976) have recently reported that human duodenal juice has an active *a*-tocopheryl acetate hydrolysing enzyme and have shown that natural bile salts (but not synthetic solubilisers) are essential for the activity of this enzyme. Results of our present study would confirm the above report regarding the essentiality of bile salts for the vitamin E esterase activity. Newmark *et al.* (1975) have also suggested that the vitamin E acetate hydrolysis is the rate limiting step in the absorption of α-tocopheryl acetate in dogs.

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