ON STEROIDS. CLVII.*

SAPONIFICATION OF ESTERS OF STEROID ACIDS AND SOME OTHER ESTERS BY POTATO TUBER SLICES AND SOME OTHER PLANT TISSUES

NGUYEN GIA CHAN and Ž.PROCHÁZKA

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

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Using a simple test the saponification ability of the esterase complex from potatoes for various esters of predominantly steroid acids was determined. Several other plants were also tested on the presence of esterases saponifying methyl deoxycholate; the relationship between the structure and the rate of saponification of esters, catalysed by the esterase complex, was also studied.

During our study of the glucosylation of exogenous steroids with plants[1] we observed that methyl deoxycholate and methyl cholate were not glucosylated by potato tissue, but that they were transformed to more polar products which we identified as free acids. In view of the fact that the yields were higher with deoxycholic acid ester (diol) than with cholic acid ester (triol), we also submitted methyl 3β-hydroxy-5-cholenate to incubation with potato tuber slices, which has only one hydroxy group in the molecule. However, contrary to our expectations, no hydrolysis took place in this case. Therefore we considered it necessary to investigate the hydrolytic activity of other plants and also to submit to our saponification test a larger series of various types of esters, especially steroidal ones, and to define thus the specificity of potato esterase complex[2-5]. Therefore we first submitted methyl deoxycholate to hydrolysis with various plants. From Table I it is evident that the activity was highest in the case of a suspension of grated potato tubers, but that some other plants also contained appreciably active esterases. The hydrolytic activity of the suspension of grated potatoes may be destroyed by short boiling (3 min). The dependence of the yield on pH did not display a sharp optimum, but below pH 3 and above pH 10 the saponification yields were lower.

More than 30 various esters of steroid acids, as well as a certain number of other types of esters were submitted to saponification in order to determine the structural factors which could affect the rate or the yield of saponification (Table II).

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It was found that the length of the esterifying alcohol in the case of deoxycholates and cholates influences the yield only little and in a manner similar to that in alkaline saponification, *i.e.* with the increasing length of the esterifying alcohol the saponification rate decreased a little (compound *IX*–*XIV* and *XXIV*, *XXV*). Similarly, isopropyl ester was saponified enzymatically slower than n-propyl ester. In contrast to this, when the side chain of deoxycholic acid was shortenend, a rapid decrease in saponification yield took place (*IX*, *XVIII*, *XIX*); for compound *XIX*, with the carboxyl group on the secondary carbon atom the yield fell to zero. Further from our experiments it follows that the hydrolysis yield is to a certain extent correlated with the number of oxygen functions (hydroxy and oxo groups) in the molecule. Esters with one oxygen function on the skeleton were saponified slowest (*I*–*VIII* and *XXIX* to *XXXI*), dihydroxy esters were saponified most easily (*IX*–*XIV* and *XX*–*XXIII*), while the saponification rate of dioxo, trioxo and trihydroxy derivatives was in between. As is evident from the results obtained with substances *IX* and *XX*–*XXIII*, the mutual position of the hydroxy groups and the ester group of dihydroxy derivatives does not play any important role. However, we observed an appreciable difference in the yields in the case of the two isomeric monohydroxy esters, *VII* and *VIII*, which by their polarity during chromatography show almost no difference. This led

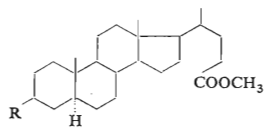
TABLE I

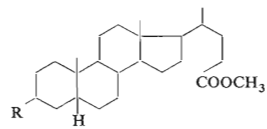
Hydrolytic Activity of Some Plants Tissue Slices as Tested with Methyl Deoxycholate

Plant	Yield of free acid
Potato, *Solanum tuberosum* L., (tubers)	++++
(shoots)	++
Apple, *Malus silvestris* (L.) MILL (fruit)	+++
Pineapple, *Ananas sativus* SCHULTZ	+++
Celery, *Apium graveolens* L. (bulb)	++
Cauliflower, *Brassica oleraces* L. var. *botrytis* L. (curb)	++
Savoy cabbage, *Brassica oleracea* L., var. *sabauda* L. (leaves)	++
Carrot, *Daucus carota* L. (root)	++
Red beet, *Beta vulgaris* L., sp. *esculenta* var. *rubra* (root)	+
Kohlrabi, *Brassica oleraces* L. var. *gongylodes* L. (bulb)	+
Pear, *Pyrus sativa* LAM. (fruit)	+
Maize, *Zea mays* L. (shoots)	+
Horse raddish, *Armoracia rusticana* L. (root)	–
Banana, *Musa sapientium* LIN. (fruit)	–
Orange, *Citrus aurantium* L. (fruit core)	–
(fruit rind)	–

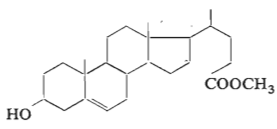
TABLE II

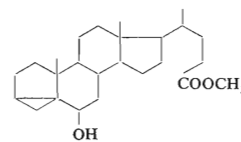
Saponification Yields[a] of Esters of Steroid Acids and Some Other Esters on Incubation with a Suspension of Grated Potato Tubers





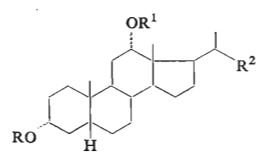
R			R		
I	—OH	+	*IV*	—OH	+
II	..OH	+	*V*	..OH	+
III	..OCOCH$_3$	+	*VI*	—OCOCH$_3$	+

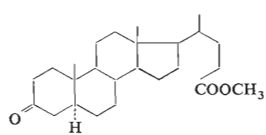




VII —

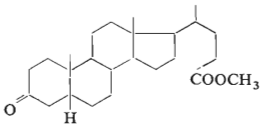
VIII ++



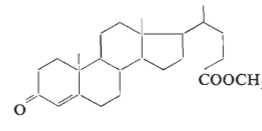


R	R^1	R^2		
			XXIX	+
IX	H	H	$(CH_2)_2COOCH_3$	++++
X	H	H	$(CH_2)_2COOC_2H_5$	++++
XI	H	H	$(CH_2)_2COOC_3H_7$(n)	+++
XII	H	H	$(CH_2)_2COOC_3H_7$(i)	++
XIII	H	H	$(CH_2)_2COOC_4H_9$(n)	+++
XIV	H	H	$(CH_2)_2COOC_5H_{11}$(n)	++
XV	CH_3CO	H	$(CH_2)_2COOCH_3$	++
XVI	H	CH_3CO	$(CH_2)_2COOCH_3$	++
XVII	CH_3CO	CH_3CO	$(CH_2)_2COOCH_3$	++
XVIII	H	H	CH_2COOCH_3	+
XIX	H	H	$COOCH_3$	—

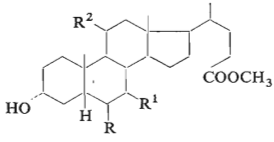
TABLE II
(*continued*)



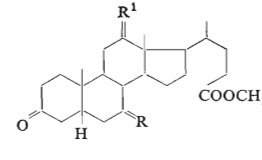
XXX +



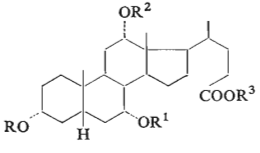
XXXI +



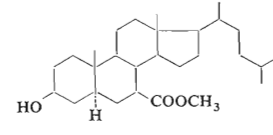
	R	R^1	R^2	
XX	..OH	H	H	++++
XXI	H	..OH	H	++++
XXII	H	H	..OH	++++
XXIII	H	H	—OH	++++



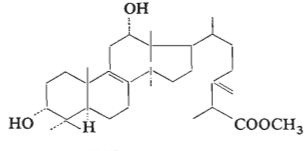
	R	R^1	
XXXII	O	H_2	++
XXXIII	H_2	O	++
XXXIV	O	O	++



	R	R^1	R^2	R^3	
XXIV	H	H	H	CH_3	++
XXV	H	H	H	n-C_4H_9	+
XXVI	H	CH_3CO	H	CH_3	++
XXVII	CH_3CO	CH_3CO	H	CH_3	++
XXVIII	CH_3CO	CH_3CO	CH_3CO	CH_3	++

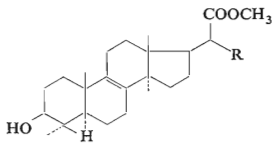


XXXV —

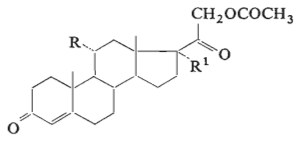


XXXVI —

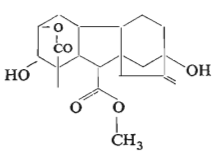
TABLE II
(*continued*)



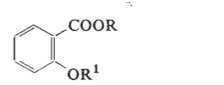
	R	
XXXVII	$(CH_3)_2CH{=}CH(CH_3)_2$	–
XXXVIII	$(CH_3)_2C{=}CH(CH_3)_2$ (=CH_2)	–



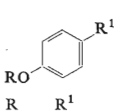
	R	R^1	
XXXIX	H	H	+++
XL	H	OH	+++
XLI[c]	$OCOCH_3$	OH	+++



XLII –



	R	R^1	
XLIII	CH_3	H	–
XLIV	H	$COCH_3$	+++
XLV	CH_3	$COCH_3$	[d]



	R	R^1	
XLVI	H	$COOCH_3$	–
XLVII	CH_3	$COOCH_3$	–
XLVIII	CH_3	$CH{=}CHCOOCH_3$	+++

IL	$CH_3(CH_2)_{16}COOCH_3$	–
L	$CH_3(CH_2)_5CH(OH)CH_2{-}CH{=}CH(CH_2)_7COOCH_3$	–
LI	tallow	–
LII	castor oil	–

[a] Four crosses mean that the starting substance disappeared almost completely and that the spot of the free acid was very strong. An approximately 40–50% yield is expressed by two crosses, *etc.* [b] Prepared by oxidation of methyl lithocholate with Jones reagent, m.p. 120 to 122°C; lit.[7] m.p. 116–120°C; [c] 21-Acetoxy group was saponified only; [d] Acetoxy group (OR^1) was saponified only.

us to the idea that mere polarity or solubility in water is not the sole factor playing a role during saponification.

During the incubation of partly or completely acetylated deoxycholic acid esters (*XV–XVII*) or cholic acid esters (*XXVI–XXVIII*) we observed on the chromatograms of the reaction products that a partial saponification of the acetyl groups also took place. Therefore we submitted three additional steroid acetates *XXXIX* to *XLI*) to saponification test. In the last three cases saponification of the primary acetoxy groups took place, while the secondary acetyl group of compound *XLI* was not saponified under the mentioned conditions. Saponification of substance *XLI* with sodium hydrogen carbonate (5%) under the conditions of the test also took place at the position 21 only, but with lower yield, while the saponification with 0·1M sodium hydroxide gave place to degradation of the substance under the same conditions.

In order to have a more complete picture of the studied esterases from potatoes, we submitted other esters to the saponification as well. Thus, for example, methyl esters of triterpenoid acids *XXXVI–XXXVIII*, substance *XXXV* and gibberellic acid GA_3 (*XLII*) were not saponified. Neither did saponification take place in the case of higher triglycerides (*LI* and *LII*) or methyl stearate or ricinoleate (*IL*, *L*), which is probably due to their insolubility.

In the series of some simple aromatic acids we observed that the acetyl group of acetylsalicylic acid (*XLIV*) hydrolysed excellently[2], while methyl salicylate (*XLIII*) remained practically unsaponified. Methyl acetylsalicylate (*XLV*) also was not saponified to free salicylic acid, but only to methyl salicylate (*XLIII*). Methyl esters of *p*-hydroxybenzoic (*XLVI*) and *p*-methoxybenzoic (*XLVII*) acids were not saponified either, while methyl *p*-methoxycinnamate (*XLVIIII*) was easily saponified. Methyl *p*-hydroxycinnamate was transformed by potato tuber tissue quantitatively in a different manner which was not investigated. On incubation with 0·1M-NaOH esters *XLIII*, *XLV* and *XLVII* were saponified in high yield (at the same temperature and reaction time as with esterases).

The esterase system from potatoes has a relatively limited specificity; it practically does not saponify esters of carboxylic acids with the carboxyl group on a secondary carbon atom or on an aromatic ring, *i.e.* on a branched carbon. Otherwise it saponified most various types of esters.

According to negative experiments with the saponification of fats with potato tissue and according to experiments aiming at the isolation and purification of the mentioned esterase complex from potatoes it follows that it is not a lipase, but an enzyme (or enzymes), which is not bound to the cell structure or particles. The preparation is relatively stable. It was possible to prepare a cell-free preparation which gave similar qualitative results as the suspension of grated potatoes itself.

In view of the fact that the presence of esterases in potato tubers is known[2–5] it would be useful to investigate the preparative isolation of this enzymatic complex or even of single isoenzymes in pure state, and to study their possible application in synthetic organic chemistry with respect to their ability to saponify ester bonds in the cold at practically neutral pH (about 5), and with respect to their selectivity for esters of acids with the carboxyl group on the primary carbon atom.

EXPERIMENTAL

The majority of the tested substances was already described and they were obtained either by purchase, donation, or preparation according to literature. The preparation of homologous esters of deoxycholic acid was carried out by esterification with corresponding alcohols in the presence of Dowex 50 WX 2 (in H^+ form) as catalyst. Higher ester could not be brought to crystallisation.

Preparative Enzymatic Saponification of Methyl Deoxycholate with Potato Tuber Slices

Two kg of peeled potatoes were coarsely grated and suspended in 10 litres of distilled water. A solution of 800 mg of methyl deoxycholate in 80 ml of methanol was added slowly and the suspension was stirred with a stream of air for 20 hours and filtered. The filtrate was adjusted to pH 3 and extracted three times with 10 l portions of chloroform or twice with 3 l of ethyl acetate. The extract was dried and evaporated to dryness. The residue was dissolved in 50 ml of chloroform and extracted five times with 20 ml portions of 5% sodium hydrogen carbonate solution. The combined hydrogen carbonate extracts were acidified with hydrochloric acid to pH 2—3 and the separated deoxycholic acid was extracted with five 100 ml portions of a chloroform–ether mixture (1 : 1, v/v). The organic extract was washed twice with 30 ml of water, dried and concentrated to dryness. The residue (0·6 g) was chromatographed on 100 g (dry column) of silica gel (60—120 μ), first with pure chloroform (one litre), then with chloroform–methanol mixture with increasing methanol content (from 1—5%, 1·5 l) and eventually with a chloroform–methanol–acetic acid mixture (95 : 5 : 1; 1·5 l). Fractions which according to analysis on thin layers contained a substance corresponding to deoxycholic acid were combined and evaporated to dryness (0·21 g). After crystallisation from ethyl acetate the product had m.p. 171—172°C and melted undepressed on admixture of authentic deoxycholic acid. IR spectra of both substances were identical and elemental analysis was satisfactory.

In a similar manner methyl cholate was also saponified and free acid isolated, but in lower yield. M.p. 199—201°C; mixture melting point with an authentic sample was undepressed. IR spectra (KBr), R_F values and elemental analysis also agreed.

Saponification Test

The tested ester (3 mg), dissolved in 0·3 ml of methanol, was added dropwise to a stirred suspension of freshly grated potato tubers (10 g) or other vegetal material in 75 ml of distilled water. The reaction mixture was shaken on a reciprocal shaker for 20—24 h, then decanted, and the liquid acidified with 0·1—0·2 ml of conc. hydrochloric acid. The decanted aqueous solution was then extracted with three 75 ml portions of chloroform. The combined extracts were dried and evaporated to dryness. The residue was dissolved in 0·15 ml of acetone and chromatographed (10 or 20 μl) on silica gel G. Most often the system chloroform–methanol–acetic acid in the 94 : 6 : 0·5 ratio was employed. Detection was carried out by spraying with conc. sulfuric acid and strong heating. The saponification yield was estimated qualitatively, by comparison with standards, on the basis of the size and the intensity of the spots of the starting ester and the formed acid. Steroidal dihydroxy and trihydroxy esters and their acetates gave yellow to brown spots (with yellow fluorescence) before carbonisation. For the detection of keto acids a dinitrophenylhydrazine solutions in methanol acidified with hydrochloric acid was used. Aromatic substances were detected under the UV light.

Methyl 6β-Hydroxy-3α,5-cyclo-5α-cholanate (*VIII*)

Methyl 3β-*p*-toluenesulfonyloxy-5-cholenate[6,7] (5 g) was dissolved in acetone (50 ml) and a solution of potassium acetate (4·5 g) in water (50 ml) was added to it and the mixture refluxed for 10 h. After cooling the mixture was extracted with ether (2 × 50 ml). The non-crystalline residue (4·2 g) obtained on evaporation of ether was chromatographed on silica gel (60—120 μ, 500 g, dry column) with a mixture of light petroleum and ether (6 : 4). After crystallisation from ether 0·8 g (22·4%) of the required methyl ester were obtained, m.p. 97—100°C, $[\alpha]_D$ +44° (*c* 1·74, chloroform). For $C_{25}H_{40}O_3$ (388·6) calculated: 77·27% C, 10·38% H; found: 77·99% C, 10·53% H.

Isolation of Esterase Fractions from Potatoes

Potato juice (1 650 ml) expressed from 4·5 g of tubers was acidified with 0·5N acetic acid to pH 5—5·5 and centrifuged. The supernatant was saturated up to 35% with ammonium sulfate and the obtained precipitate (a small amount) was separated by centrifugation. The supernatant was then saturated with ammonium sulfate up to 55% and stored in a refrigerator overnight. The rich precipitate was separated by centrifugation and the supernatant saturated with ammonium sulfate up to 75% saturation. All three protein fractions were freeze-dried and used for the saponification test. Instead of using the 10 g of grated potatoes 0·5 g of the enzyme preparation were suspended in water. The result, according to a visual evaluation of the intensity of spots, was similar with all three precipitates to the test with fresh potato tubers. When the preparation obtained by saturation with ammonium sulfate up to 55% was reprecipitated with acetone, the obtained powder was approximately equally active.

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