A Fast, Sensitive HPLC Method for the Determination of Esterase Activity on α-Tocopheryl Acetate

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Abstract

Although data on the behavior of tocopheryl acetate (Tac) in the gut is rare, some studies show that this ester is hydrolyzed in the intestine and the released tocopherol (Tol) is absorbed. An HPLC method is published for the simultaneous analysis of Tol and Tac in mixed micelles. This study shows how this method is used for the measurement of esterase activity on Tac using commercial cholesterol esterase. The rate of hydrolysis is determined in less than 20 min on the basis of both Tac disappearance and Tol appearance. The present method is useful for investigating esterase activities on Tac in model systems that mimick the absorption environment or in any biological or nonbiological medium.

Introduction

Vitamin E is widely used as a supplement in animal and human diets, as well as in pharmaceutic and cosmetic sectors. It is generally given in the form of DL-α-tocopheryl acetate (Tac), in which the reactive hydroxyl group is acetylated to prevent its oxidation under storage and digestion conditions. On the other hand, according to the general concensus, only the alcohol form (i.e., the biologically active form) of vitamin E traverses the apical membranes of intestinal absorptive cells (1,2). The conversion of Tac to tocopherol (Tol) (Figure 1) may constitute a limiting factor for the biological utilization of vitamin E ester, as has been emphasized in some studies (3–5). These studies have shown the percentage of intestinal absorption of vitamin E to be related to the susceptibility of the corresponding ester to hydrolysis by pancreatic esterase. The enzyme identified was cholesterol esterase (EC 3.1.1.13) (6), although an esterase activity of intestinal origin has also been described (7).

The hydrolysis of Tac by this enzyme (provided mainly in the pancreatic juice) has been investigated in micellar solutions based on sodium taurocholate (4,7), on sodium cholate (5,8), or whole bile secretions (3). These mediums, more or less, mimic the conditions of the intestinal environment with regard to bile salts.

For enzymatic activity measurement, the Emmerie–Engel colorimetic method (9), which is based on the reducing properties of the Tol released, has been widely used for this purpose (4,7). Titrimetry (6), radiolabeling, and thin-layer chromatography were also used (3). More recently, Jensen et al. and Lauridsen et al. used the high-performance liquid chromatographcy (HPLC) method when comparing the biopotency of Tac and tocopheryl sussinate in broilers (5) and studying the effects of bile acids on the hydrolysis of retinyl and tocopheryl esters (10). Tol was determined by Jensen et al. and Lauridsen et al. using methods based on extraction techniques with heptane and fluorescence detection after a saponification step. These methods suffered from the disadvantage of having low specificity and precision, and they did not permit Tol oxidation to be estimated during extraction and analysis.

An HPLC method for the simultaneous analysis of Tol and Tac, within less than 4 min and with low limits of quantitation in mixed micelles, was recently described (11). This study uses this method for measuring the rate of Tac hydrolysis by a commercial

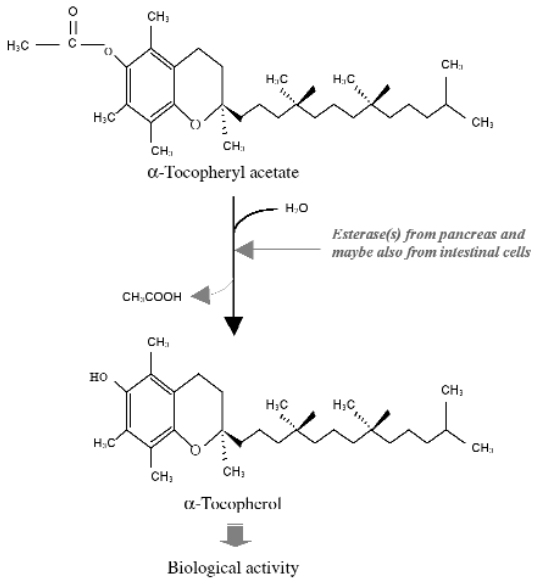

Figure 1. Conversion of Tac to Tol in the intestinal lumina.

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pancreatic cholesterol esterase. The enzymatic activity was determined within 20 min by either the disappearance of the substrate Tac or the appearance of the product Tol.

Experimental

Equipment

The HPLC system consisted of a GP40 Dionex gradient pump (Dionex, Sunnyval, CA), an AS 3500 Spectra System autosampler from Thermo Electron (Fremont, CA), and a model 486 variable wavelength detector (Waters, Milford, MA). The area under the peaks was integrated with an Olivetti P75i integrator (Olivetti, Paris, France), using the Borwin chromatography software program (JMBS, Grenoble, France). The other equipment used for solubilization and the extraction of Tac and Tol were the same as previously described (11).

Reagents

DL-α-Tol (> 98%), DL-α-Tac (98%), and sodium taurocholate (> 97%) were from Fluka (Buschs, Schweiz). Cholesterol (99%), oleic acid (99%), egg yolk L-α-lysophosphatidylcholine (99%), 1-monopalmitoyl glycerol, retinyl acetate, and porcine cholesterol esterase were purchased from Sigma (Saint Quentin, France). According to the supplier, the enzyme powder contained 70% protein and 43 units/mg of protein, with one unit being the amount of enzyme needed for the hydrolysis of 1 µmole of cholesteryl oleate per min. The solvents and the handling conditions were as previously reported (11).

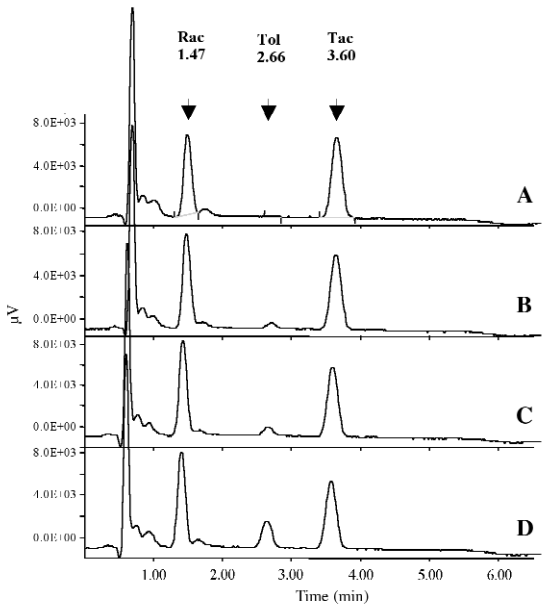

Figure 2. Time course of the hydrolysis of tocopheryl acetate by cholesterol esterase. Cholesterol esterase (0.75 U/mL, Sigma C9464) was incubated with Tac (0.1mM) in mixed micelle solution (see Enzyme assay section). At various periods of time, 150 µL of the reaction mixture was sampled, and vitamin E was extracted and analyzed as previously described (11). The reaction times were; 0, (A); 30, (B); 60, (C); and 120 min, (D). The internal standard was retinyl acetate (Rac).

Enzyme assay

The enzyme powder was solubilized in deionized water and stored at –20°C until used. The protein concentration was determined according to Bradford (12). The hydrolysis of Tac was achieved at 37°C in a micellar solution formed by a phosphate buffer (35mM, pH 6.5, NaCl 0.15M), which contained sodium taurocholate (4mM), oleic acid (0.4mM), and 0.1mM each of monopalmitine, lysophophatidylcholine, cholesterol, and Tac (11). Using 1.5-mL colored microcentrifuge tubes, which were placed in a thermostated bath, the reaction was started by mixing the enzyme solution (25 µL) with the mixed micelles (475 µL) using vigorous vortexing (5 s). After fixed periods of time, 150 µL of the micellar solution was undertaken, and the reaction was stopped by adding 150 µL of ethanol [containing retinyl acetate (internal standard)] and vigorous vortexing (5 s). In the blank, the reaction stopped immediately after the addition of the enzyme. Tac and Tol were extracted using an ethanol–hexane drying procedure and detected at 284 nm, as previously described (11).

Results and Discussion

Figure 2 shows typical chromatograms of the time course conversion of Tac to Tol. In the blank and the three kinetic points, the sum of Tac and Tol on a molar basis was close to 100% of the incubated ester, whereas the retinyl acetate peak was constant in all four HPLC runs. In the blank (time zero), the reaction stopped immediately after mixing the enzyme with the substrate because the recovery of Tac and Tol changed, to a greater or lesser degree, as a result of more or less important variables, such as, for example, water or even other proteins being added instead of the enzyme solution (data not shown). The chromatographic runs in Figure 2 are set at 6.5 min and may be shortened by reducing the column equilibration step (2.5 min). Consequently, the rate of Tac hydrolysis may be determined in three to four runs, as long as the total does not exceed 20 min. The present method had advantages over previous methods. First, there was no risk of interference by compounds reducing with Tol, as was possible with the Emmery–Engle reductometric method. Second, it does not require the specific equipment

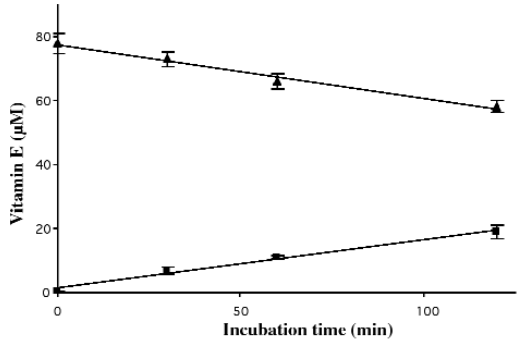

Figure 3. Kinetic plots of Tac disappearance and Tol appearance after hydrolysis of Tac by cholesterol esterase. The kinetic points correspond to the mean values of two determinations.

needed for radiolabeling, thin-layer chromatography (3), or gas chromatography–mass spectrometry (8) methods. Third, as compared with the HPLC method of Jensen et al. (5), less time and organic solvent was consumed, and there was no need for saponification because simultaneous determination of Tac and Tol was performed.

The kinetic plots of Tac disappearance and Tol appearance are shown in Figure 3. The regression equation of Tac disappearance is:

$$y = -0.169x + 77.6 \quad (R^2 = 0.987) \qquad \text{Eq. 1}$$

whereas that of Tol appearance is:

$$y = 0.151x + 1.4 \quad (R^2 = 0.985) \qquad \text{Eq. 2}$$

In the kinetic plot of Tac disappearance the ordinate at the abscissa origin 77.6 corresponds to 77.6µM (77.6% of Tac recovery). The value was slightly higher than previously reported for similar concentrations of Tac (11), suggesting an emulsifying effect of the proteins added to the mixed micelles, which enhanced Tac extraction recovery. In the kinetic plot of Tol appearance, the ordinate at the origin (1.4µM) was close to the limit of quantitation of Tol in the mixed micelles (11). It should also be noted that the slope of Tol appearance was approximately 10% lower than that of Tac disappearance, suggesting some oxidation of Tol under the present experimental conditions.

From the kinetic view point based on Tac disappearance, the specific activity of the used commercial enzyme was 9.4 nmoles of Tac hydrolyzed per min per mg of proteins. According to Sigma, the value was much higher when performed on cholesteryl oleate (43 units per mg of proteins) or when performed using rat pancreatic juice on Tac (measured titrimetrically) [47 µmoles of Tac hydrolyzed per min per mg of protein, (6)]. But the value was quite similar to those reported by Mathias et al. (7), using Tac as the substrate and the reductometric Emmery–Engle method (10 and 3.3 nmoles of Tac hydrolyzed per min per mg of proteins from the intestinal and pancreatic secretions, respectively). Therefore, the question should be raised about the large differences in these specific activity values, especially between those performed on the same substrate. In the latter case, the analytical methods and the degree of purity of the enzyme most likely account for the difference between values.

Conclusion

The latest report of Food and Nutrition Board (13), concerning vitamin E utilization, placed an emphasis on the lack of information on the absorption mechanism of vitamin E in the gut. Using Tac as a source of vitamin E, in line with its hydrolysis, was not considered in this report, most likely because of the scarceness of data on the topic. The present report provides analytical means for screening esterase activity on Tac in a medium mimicking the intestinal milieu. Such means may also serve for the purification of esterase activity for both Tac and any tocopheryl ester, provided the experimental conditions are adapted. More information should, therefore, be supplied concerning the absorption of these tocopheryl esters, supported by mechanistic biochemical events.

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References

1. D.W. Nierenberg and D.C. Lester. Determination of vitamins A and E in serum and plasma using a simplified clarification method and high-performance liquid chromatography. *J. Chromatogr. B* **345:** 275–84 (1985).
2. M.G. Traber. Utilization of vitamin E. *Biofactors* **10:** 115–20 (1999).
3. T. Nakamura, Y. Aoyama, T. Fujita, and G. Katsui. Studies on tocopherol derivatives: V. Intestinal absorption of several d,1-3,4-$^3H_2$-alpha-tocopheryl esters in the rat. *Lipids* **10:** 627–33 (1975).
4. D.P. Muller, J.A. Manning, P.M. Mathias, and J.T. Harries. Studies on the intestinal hydrolysis of tocopheryl esters. *Int. J. Vitam. Nutr. Res.* **46:** 207–10 (1976).
5. S.K. Jensen, R.M. Engberg, and M.S. Hedemann. All-rac-alpha-tocopherol acetate is a better vitamin E source than all-rac-alpha-tocopherol succinate for broilers. *J. Nutr.* **129:** 1355–60 (1999).
6. D. Lombardo and O. Guy. Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. II. Action on cholesterol esters and lipid-soluble vitamin esters. *Biochim. Biophys. Acta* **611:** 147–55 (1980).
7. P.M. Mathias, J.T. Harries, T.J. Peters, and D.P.R. Muller. Studies on the *in vivo* absorption of micellar solutions of tocopherol and tocopheryl acetate in the rat: demonstration and partial characterization of a mucosal esterase localized to the endoplasmic reticulum of the enterocyte. *J. Lipid. Res.* **22:** 829–37 (1981).
8. H.A. Zahalka, S.C. Shee, C. Cheng, G.W. Burton, and K.U. Ingold. Hydrolysis of stereoisomeric alpha-tocopheryl acetates catalyzed by bovine cholesterol esterase. *Biochim. Biophys. Acta* **921:** 481–5 (1987).
9. A. Emmerie and C. Engel. Colorimetric determination of alpha-tocopherol (vitamin E). *Rec. Trav. Chim.* 1351–55 (1938).
10. C. Lauridsen, M.S. Hedemann, and S.K. Jensen. Hydrolysis of tocopheryl and retinyl esters by porcine carboxyl ester hydrolase is affected by their carboxylate moiety and bile acids. *J. Nutr. Biochem.* **12:** 219–24 (2001).
11. S. Castan, C. Villard, S. Jakob, A. Puigserver, and E.H. Ajandouz. A fast, sensitive method for the simultaneous determination of a-tocopherol and a-tocopheryl acetate in mixed micelles. *J. Chromatogr. B* **822**: 339–346 (2005).
12. M.M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–54 (1976).
13. Food and Nutrition Board. *Report of the Panel on Dietary Antioxidants and Related Compounds*. Institut of Medicine, National Academy Press, Washington, DC. 2000, P. 186–283.

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