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Oxidation reactions of steryl esters in a saturated lipid matrix

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ABSTRACT

In the present study, the formation and further reactions of intact steryl ester hydroperoxides were followed in a tripalmitin matrix maintained at 100 °C. The effects of the acyl moiety and its unsaturation degree, and of the sterol structure were investigated. Intact steryl ester hydroperoxides were isolated from the lipid matrix by a developed solid-phase extraction (SPE) method and were determined by HPLC–ELSD. Further reactions of hydroperoxides were followed by determining secondary oxidation products of sterol by GC-FID/MS and oligomers by HPSEC-RI. The oxidation of sterol decreased when its solubility in the lipid medium was increased by introduction of acyl moiety. Increased unsaturation of the acyl or steryl moiety of steryl ester shortened the induction period and increased the oxidation of both steryl and acyl moieties. Thus, by changing the chemical and physical properties of sterols, their oxidation may be greatly affected.

1. Introduction

In food products, functional lipid components, such as plant sterols, are present in a complex mixture of other compounds. The presence and reactions of these surrounding compounds also affect the reactions of the functional lipids. In oxidation reactions, other lipids function both as hydrogen sources and as radical formers in radical chain reactions (Labuza & Dugan, 1971; Schaich, 2005). As these other components participate in the oxidation reactions, they not only affect the rate of oxidation, but they also may modify the profile of the formed products. For example, in lipid model systems, triacylglycerols were suggested to induce the oxidation of free cholesterol at 25–180 °C (Kim & Nawar, 1991; Li, Ohshima, Shozen, Ushio, & Koizumi, 1994; Nawar, Kim, Li, & Vajdi, 1991). Free fatty acids, on the other hand, not only increased the oxidation of cholesterol, but also changed the profile of the formed oxidation products (Kim & Nawar, 1991; Xu, Sun, Liang, Yang, & Chen, 2011).

Previous oxidation studies conducted on plant sterols and their fatty acyl esters in lipid matrices have focused on the oxidation reactions of the steryl moiety (Yanishlieva, Marinova, Schiller, & Seher, 1985; Blekas & Boskou, 1989; Soupas, Juntunen, Lampi, & Piironen, 2004; Soupas, Huikko, Lampi, & Piironen, 2005) or on the changes occurring within the matrix (Lampi, Dimberg, & Kamal-Eldin, 1999; Winkler & Warner, 2008). In a previous study, a more complete oxidation was found for the steryl moiety in steryl esters having rapeseed oil acyl moieties (7.5% saturated, 66% monounsaturated, 26% polyunsaturated) than for the corresponding free sterols maintained in a saturated lipid matrix at moderate temperature (100 °C) (Soupas et al., 2005). However, at elevated temperatures (180 °C) the opposite situation occurred. The oxidation of the steryl moiety was greater for free sterols than for esterified sterols. Plant sterols with an ethyldiene group in the steryl side chain, such as Δ5-avenasterol, have been suggested to act as antioxidants and antipolymerising agents in frying oils (Blekas & Boskou, 2010). Therefore, monitoring the changes occurring in lipid matrices during prolonged heating has been of particular interest. A similar efficiency has been suggested for free sterols and corresponding fatty acyl esters in preventing the degradation of frying oils (Blekas & Boskou, 2010).

The oxidation of steryl esters in lipid media has been previously studied by measuring peroxide values and by determining the secondary oxidation products of sterol after saponification (Yanishlieva et al., 1985; Blekas & Boskou, 1989; Soupas et al. 2004, 2005; Tabee, Azadmard-Damirchi, Jagerstad, & Dutta, 2008). Peroxide values reflect all hydroperoxyl groups present in the sample, thus they measure the oxidation of the matrix and not only of the sterols. Secondary oxides of sterol, on the other hand, reflect only the oxidation of the steryl moiety in steryl esters. Moreover, information about the initial oxidation is not gained. Previously, the

Abbreviations: CIS-MS, coordination ion spray mass spectrometry; DAD, diode-array detector; ELSD, evaporative light-scattering detector; Fa-OOH, acyl moiety hydroperoxide; HPSEC, high-performance size-exclusion chromatography; MTBE, methyl-*tert*-butyl ether; PV, peroxide value; RI, refractive index; SiOH, silica; SPE, solid-phase extraction; St-OOH, steryl moiety hydroperoxide; THF, tetrahydrofuran.

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primary oxidation of steryl esters, determined as intact molecules, showed that when neat steryl esters were oxidised at 100 °C, an increased unsaturation of the acyl moiety increased the oxidation of both the steryl and the acyl moieties (Lehtonen, Lampi, Ollilainen, Struijs, & Piironen, 2011). Both the steryl and acyl moiety hydroperoxides were detected even in the initial state and they were formed concomitantly. However, the formation and reactions of steryl ester hydroperoxides, determined as intact molecules, have not been followed in lipid matrices. Since the matrix may alter the oxidation rate and may influence the oxidation pathways of sterols, widening the knowledge of steryl ester oxidation within lipid matrices becomes important.

The aim of this study was to investigate the formation and reactions of steryl ester hydroperoxides in a lipid matrix. A solid-phase extraction (SPE) method was developed for isolatation of steryl ester hydroperoxides as intact molecules from a lipid matrix. This method, in combination with HPLC–ELSD, allowed investigation of the effects of esterification, unsaturation of the acyl moiety and sterol structure on the formation of monohydroperoxides, by comparing the oxidation of cholesteryl stearate, oleate and linoleate, stigmasteryl oleate and free cholesterol. Peroxide values were also determined and further reactions of the hydroperoxides were followed by determining secondary oxidation products of sterols by GC-FID/MS and oligomers by HPSEC-RI.

2. Materials and methods

2.1. Materials and reagents

The synthesis of stigmasteryl oleate was performed with stigmasterol (24S-ethylcholest-5,22-dien-3β-ol; ⩾95%; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and oleic acid (9-Octadecenoic acid; ⩾99%; Nu-Chek Prep, Elysian, MN, USA) in the presence of *Candida Rugosa* lipase (type VII; ⩾700 unit/mg solid; Sigma-Aldrich Chemie GmbH). Cholesteryl stearate (Cholest-5-en-3β-yl octadecanoate; ⩾99%), cholestryl oleate (Cholest-5-en-3β-yl 9-octadecenoate; ⩾99%) and cholesteryl linoleate (Cholest-5-en-3β-yl 9,12-octadecadienoate; ⩾99%) used in the oxidation studies were purchased from Nu-Chek Prep. Tripalmitin (1,2,3-triyl trihexadecanoate; ⩾85%) and free cholesterol (Cholest-5-en-3β-ol; ⩾99%) were acquired from Sigma-Aldrich Chemie GmbH. Iron(III)chloride ($FeCl_3$, Titrisol®; Merck, Darmstadt, Germany), iron(II)chloride-4-hydrate ($FeCl_2$ $4H_2O$; Fluka Chemie, Buchs, Switzerland) and ammonium thiocyanate (NH_4SCN) (Riedel-de Haën, Seelze, Germany) were used for the determination of peroxide values. Cold saponification of the oxidised samples was performed in ethanol (99.5%; ALTIA, Rajamäki, Finland) with saturated potassiumhydroxide (KOH; Merck) solution prepared in MilliQ water (Millipore, Mosheim, France). Cholest-5-ene-3β,19-diol (19-OH-cholesterol) served as an internal standard in the determination of the secondary oxides of sterol. The hydroxysterols were silylated for the GC-FID/MS analysis with a 99:1-mixture (*V*/*V*) of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; >98%; Merck) and trimethylchlorosilane (TMCS; 99%; Fluka Chemie) in anhydrous pyridine (>99%; Sigma-Aldrich Chemie). All the used solvents were HPLC grade: diethyl ether (DEE) and methanol (MeOH) (J.T. Baker, Deventer, The Netherlands), 1-decanol (⩾95%; Fluka Chemie), dichloromethane, ethyl acetate (EtAc), heptane, methyl-*tert*-butyl ether (MTBE), 2-propanol (IPA) and tetrahydrofuran (THF) (Rathburn Chemicals Ltd., Walkerburn, Scotland).

2.2. Synthesis of stigmasteryl oleate

Enzyme catalysed synthesis of steryl esters enables the use of mild reaction conditions and therefore the formation of oxidation products and other artefacts may be avoided. Moreover, the removal of catalyst is easy. Therefore, stigmasteryl oleate was synthesised using a well studied *Candida Rugosa* lipase according to Kim and Akoh (2007), after some modifications. For the synthesis, a 50 mg sample of stigmasterol (⩾95%) and 72 mg of oleic acid (⩾99%) were mixed with 8.8 mg of lipase (*Candida Rugosa*, type VII, ⩾700 unit/mg solid) and 1.5 ml of heptane. The mixture was maintained at 50 °C for 17 h with gentle shaking. After cooling, the steryl esters formed in the reaction mixture were extracted three times with 3 ml of diethyl ether. The combined extract was dried with anhydrous Na_2SO_4, filtered, and the solvent was evaporated to dryness under a N_2 flow at 30 °C. The residue was dissolved in 10 ml of heptane for immediate purification by SPE. The final yield of stigmasteryl oleate was 38% and purity was 96%. The preparation contained 4% of other plant steryl oleates.

2.3. Purification of steryl esters and tripalmitin

Purity of each steryl ester preparations was assured by solid-phase extraction (SPE), as described earlier (Lehtonen, Lampi, Ollilainen et al., 2011). Briefly, 100 mg of steryl ester was purified on a 5 g SiOH-SPE cartridge (Strata SI-1 Silica, Phenomenex, Inc.; Torrance, CA, USA) using 2 × 20 ml of heptane. The ester was collected with 2 × 20 ml of heptane/MTBE (98:2, *V*/*V*). The remaining solvent was evaporated and the preparation was stored in heptane at −20 °C for further use.

Tripalmitin was purified by adsorption chromatography according to a previously described method (Lampi et al., 1999), after slight modifications. Briefly, 100 g of tripalmitin was passed through a glass column packed with 250 g of activated aluminium oxide (Al_2O_3) using 200 ml of heptane/dichloromethane (1:1, *V*/*V*). Purified tripalmitin contained 4% of other saturated acyls.

The amounts and purities of the steryl esters were determined by GC-FID/MS (Lampi, Juntunen, Toivo, & Piironen, 2002). The lipid profiles of the steryl esters and tripalmitin were determined by TLC (SiOH-plate; eluent: 14:1 heptane/ethyl acetate, *V*/*V*). The presence of hydroperoxides was monitored by HPLC–ELSD (Lehtonen, Kemmo, Lampi, & Piironen, 2011; Lehtonen, Lampi, Ollilainen et al., 2011) and the presence of tocopherols by HPLC-FLD (Ryynänen, Lampi, Salo-Väänänen, Ollilainen, & Piironen, 2004). The acyl composition was determined by GC-FID (Soupas et al., 2005). No other lipids, oxidation products or tocopherols were detected in the steryl ester preparations or tripalmitin by the used methods.

2.4. Oxidation of steryl esters and free sterol in the tripalmitin matrix

Oxidation of cholesteryl stearate, oleate and linoleate, stigmasteryl oleate and free cholesterol were carried out in tripalmitin. For autoxidation, 92 mg of tripalmitin and 12 mg of steryl ester (corresponding to 8 mg of sterol) or 8 mg of free cholesterol were mixed well and placed in a glass vial (22 × 46 mm). The solvent was evaporated gently under a N_2 stream (+30 °C) to create a uniform sample film. The sample was maintained in an open vial in the dark at 100 °C for 0–5 days. After this oxidation treatment, the sample was cooled in a desiccator and dissolved in 5 ml of heptane/dichloromethane (1:1, *V*/*V*). For each time point, three replicate samples were oxidised and the experiment was repeated two times ($n = 6$).

2.5. Isolation of steryl ester and free sterol hydroperoxides by SPE

A solid-phase extraction (SPE) method was developed for the isolation of intact steryl ester hydroperoxides and free sterol hydroperoxides from a lipid matrix. Silica (SiOH) and aluminium oxide (Al_2O_3) sorbents were initially screened for this isolation. Silica was chosen for further development since the hydroperoxides tended to reduce to hydroxides on the Al_2O_3. Mixtures of heptane