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Impact of thermooxidation of phytosteryl and phytostanyl fatty acid esters on cholesterol micellarization in vitro

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ABSTRACT

The effects of thermooxidation of a phytosteryl/-stanyl and a phytostanyl fatty acid ester mixture on cholesterol micellarization were investigated using an in vitro digestion model simulating enzymatic hydrolysis by cholesterol esterase and subsequent competition of the liberated phytosterols/-stanols with cholesterol for incorporation into mixed micelles. As a first step, relationships between different doses of the ester mixtures and the resulting micellarized cholesterol were established. Subsequent subjection of the thermooxidized ester mixtures to the in vitro digestion model resulted in three principal observations: (i) thermal treatment of the ester mixtures led to substantial decreases of the intact esters, (ii) in vitro digestion of cholesterol in the presence of the thermooxidized ester mixtures resulted in significant increases of cholesterol micellarization, and (iii) the extents of the observed effects on cholesterol micellarization were strongly associated to the remaining contents of intact esters. The loss of efficacy to inhibit cholesterol micellarization due to thermally induced losses of intact esters corresponded to a loss of efficacy that would have been induced by an actual removal of these amounts of esters prior to the in vitro digestion. The obtained results suggest that in particular oxidative modifications of the fatty acid moieties might be responsible for the observed increases of cholesterol micellarization.

1. Introduction

An elevated plasma low-density lipoprotein (LDL)-cholesterol level is an acknowledged factor contributing to the development of cardiovascular diseases [1]. A daily dietary intake of 2–3 g phytosterols/-stanols has been proven to effectively reduce increased LDL-cholesterol concentrations by 9–12% based on a reduction of intestinal cholesterol absorption [2–5]. Therefore, phytosterols/-stanols are commercially being added to a broad spectrum of foods, commonly as esters of plant oil fatty acids [6]. The consumption of these foods offers a convenient alternative for hypercholesterolemic patients as compared to drug use or restriction of dietary cholesterol intake. The ester preparations are specified and may either consist of phytostanyl fatty acid esters (PSTA-FA), or of mixtures of both phytostanyl and phytosteryl fatty acid esters (PSTE-FA) [7,8].

The intestinal digestion of dietary consumed plant steryl/stanyl fatty acid esters involves their pre-emulsification into lipid droplets and enzymatic hydrolysis by pancreatic cholesterol esterase [9–11]. Subsequently, similarly to cholesterol, the released plant sterols/stanols are incorporated into dietary mixed micelles, composed of enzymatically hydrolyzed lipids and bile salts acting as emulsifiers [12,13]. The mixed micelles are able to interact with the apical brush border membrane, making the incorporated sterols/stanols available for absorption by the enterocytes [5]. Phytosterols/-stanols have been shown to be favored over cholesterol for incorporation into the mixed micelles due to their higher hydrophobicity [12]. This competition results in a significant restriction of the micellar solubilization of cholesterol and thus of the proportion of cholesterol available for absorption, which is being unequivocally acknowledged to be a major mechanism underlying the LDL-cholesterol-lowering properties of phytosterols/-stanols [14,15]. Esterified phytosterols/-stanols are probably not able to affect micellar cholesterol solubilization [16]; it has been shown that they need to be hydrolyzed to reduce intestinal cholesterol absorption in hamsters [17]. Thus, the sequence of enzymatic hydrolysis and competition of the phytosterol/-stanol moieties with cholesterol for incorporation into the mixed micelles constitutes the prerequisite for phytosteryl/-stanyl fatty acid esters added to foods to exert their cholesterol-lowering properties.

However, phytosteryl/–stanyl fatty acid esters are susceptible to oxidation reactions, particularly in the presence of light, oxygen and heat, leading to mixtures of primary, secondary, and tertiary oxidation products [18]. Both the sterol and the fatty acid moieties may be affected [19–21]. The formation and the physiological effects of the secondary oxidation products of the phytosterol moieties, the so-called phytosterol oxidation products (POPs), are being investigated due to

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potential adverse effects to human health such as pro-inflammatory and pro-atherogenic properties [22,23]. However, the balance between risks and benefits of enriched foods might change upon heating not only owing to the formation of POPs, but also due to concurrent losses of the intact, functionality-bearing phytosteryl/-stanyl fatty acid esters. As reported recently for PSTE-FA- and PSTA-FA-enriched margarines heated under household-type conditions, the contents of the intact esters significantly decreased [24]. These findings may raise concerns regarding a potentially reduced efficacy of thermooxidized ester mixtures to displace cholesterol from the mixed micelles.

There are two in vivo studies indicating a loss of anti-atherogenic properties upon oxidation of free phytosterols. Tomoyori et al. [25] showed that ApoE-deficient mice had elevated cholesterol oxidation product serum levels after having been fed a diet containing POPs instead of phytosterols. In the second study, substituting phytosterols by POPs in a diet fed to hamsters resulted in a loss of the potential to reduce aortic plaque size and aortic cholesterol levels observed for the phytosterol-enriched diet [26]. However, in these studies the phytosterols were fully replaced by the corresponding secondary oxidation products. Thus, conclusions regarding the effects of realistically heated mixtures still containing remaining intact phytosterols in addition to various types of phytosterol oxidation products cannot be drawn; beyond, the potential effects of thermooxidized mixtures of fatty acid esters remain uncertain. There is only one study available on the effects of oxidation of the esters in which the in vitro hydrolysis of two selected oxidation products of sitosteryl oleate by cholesterol esterase was investigated [27]. Oxidation of the sterol moiety favored enzymatic hydrolysis, whereas an oxidative modification of the fatty acid moiety resulted in an almost complete loss of hydrolysis of both the acyl moiety oxidation product and the intact ester. These contrary effects of different types of oxidation products underline the necessity to focus on heated mixtures of fatty acid esters, containing a realistic spectrum of intact and oxidized esters.

Therefore, the objective of the present study was to assess the effects of thermooxidation of phytosteryl/-stanyl fatty acid esters on cholesterol micellarization using an in vitro lipid digestion model simulating both enzymatic hydrolysis and final formation of mixed micelles, thereby taking into account all potential influences deriving from a heat-treated ester mixture. First, a relationship between different doses of a PSTE-FA and a PSTA-FA mixture, exhibiting profiles similar to those mixtures used for enrichment of foods, and the resulting cholesterol micellarization was established. Subsequently, the ester mixtures were subjected to thermooxidation for different time spans and the resulting effects on cholesterol micellarization were determined, complemented by analyses of the formation of POPs and the decreases of the intact esters.

2. Materials and methods

2.1. Materials

A PSTE-FA mixture ("Vegapure® 95E", containing 95.1% phytosteryl/-stanyl esters) was provided by Cognis GmbH (Illertissen, Germany). A PSTA-FA mixture ("plant stanol ester, STAEST-115", containing 93.3% phytostanyl esters) was provided by Raisio Group (Raisio, Finland). The compositions of individual esters of both mixtures are shown in the Supplementary data (Tables S1 and S2).

2.2. Chemicals

Acetic anhydride (≥99%), l-α-phosphatidylcholine from dried egg yolk (≥50%), 5,6β-epoxycholesterol (≥95%), bile acids sodium salts, calcium chloride, 5α-cholestan-3β-ol (≥95%), cholesterol (89%), cholesterol esterase from porcine pancreas (35 U/mg), cholesteryl palmitate (≥98%), diethylether (extra pure), glyceryl trioleate (> 99%), 7-ketocholesterol (≥90%), maleic acid (reagent plus®), *N,O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS), pancreatin from porcine pancreas (4 × USP specifications), potassium hydroxide (≥85%), pyridine (99.8%), sodium chloride, stigmastanol (95%), stigmasterol (~95%), and Trizma® base (≥99.9%) were obtained from Sigma-Aldrich (Steinheim, Germany). Ethyl acetate (EtOAc; Rotisolv, LC–MS) was purchased from Carl Roth (Karlsruhe, Germany). Citric acid anhydrous (for synthesis), sodium hydroxide, and sodium methoxide (30% in methanol) were purchased from Merck (Darmstadt, Germany). Chloroform (AnalaR Normapur), ethanol (99% denatured with 1% MEK), *n*-hexane (AnalaR Normapur), *n*-hexane (HiPerSolv Chromanorm), isopropanol (HiPerSolv Chromanorm), methanol (HiPerSolv Chromanorm), sodium sulfate (anhydrous) and water (HiPerSolv Chromanorm) were purchased from VWR International (Darmstadt, Germany). Methyl *tert*-butyl ether (MTBE) was supplied by Evonik Industries AG (Essen, Germany). Beta-sitosterol (~75%, with ca. 10% campesterol) was purchased from Acros Organics (Morris Plains, NJ, USA). A mixture of wood plant stanols (Reducol® Stanol Powder) was provided by Cognis GmbH (Illertissen, Germany).

2.3. Heating of phytosteryl and phytostanyl fatty acid esters

The PSTE-FA and the PSTA-FA mixtures (200.0 mg) were weighed into an 11 mL glass vial (bottom surface area: 2.01 cm^2) and heated in a pre-heated oven at 180 °C for 0.5, 1, 2, 3, and 4 h; the PSTA-FA mixture was also heated for 5 h. After cooling in a desiccator, the samples were dissolved in chloroform, a solvent shown to be suitable for the analysis of thermooxidized enriched margarines [24]. The solution was transferred to a graduated flask (final volume of 2 mL), and an aliquot of 1 mL corresponding to 100 mg of the originally used ester mixture was transferred to a 50 mL centrifuge tube. The solvent was evaporated via a gentle stream of nitrogen, and the residue was subjected to the in vitro digestion experiment. For determination of intact esters and POPs, the remaining 1 mL was transferred to a graduated flask with a final volume of 20 mL.

2.4. Analysis of intact phytosteryl and phytostanyl fatty acid esters

Analyses were performed according to previously described approaches [28,29]. Briefly, to aliquots of the heated ester mixtures corresponding to 0.25 mg originally used PSTE-FA and PSTA-FA, 50 µL of the internal standard cholesteryl palmitate [1 mg/mL] was added. For analyses of the non-heated mixtures, aliquots of stock solutions [10 mg/mL] corresponding to 0.25 mg esters were used. After evaporation of the solvent, the residue was dissolved in 1000 µL of *n*-hexane and an aliquot was subjected to an SPE procedure (NH_2-modified silica gel). The dried residue was dissolved in 150 µL of EtOAc, filtered through a 0.2 µm membrane filter, and subjected to UHPLC-APCI-MS analysis according to previously described conditions [28,29], using a 6470 triple quadrupole mass selective detector (Agilent Technologies, Waldbronn, Germany). Identification of the individual esters was based on relative retention times and mass spectral data, quantitation on the generation of calibration functions [28,29].

2.5. Analysis of phytosterol oxidation products

For analyzing the non-heated PSTE-FA, 20 mg material (accuracy of ± 0.1 mg) was directly weighed into 11 mL vials and subjected to further analysis. For analyzing the heated PSTE-FA, aliquots corresponding to 20 mg of originally used PSTE-FA were placed into 11 mL vials. To the samples, 0.0155–0.34 mg of 5,6β-epoxycholesterol (IS_1) and 0.015–0.35 mg of 7-ketocholesterol (IS_2) were added as internal standards (IS); the actual amounts were adjusted according to the expected amounts of POPs. The transesterification and subsequent acetylation were performed as previously described [30]. The residues were dissolved in 0.15–3 mL of *n*-hexane/MTBE/isopropanol (80:20:0.3 v/v/v), depending on the expected concentrations of POPs in the sample,