Effect of Lipoxygenase Oxidation on Surface Deposition of Unsaturated Fatty Acids

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ABSTRACT: We studied the interactions of lipid molecules (linoleic acid, glycerol trilinoleate and a complex mixture of wood extractives) with hydrophilic and hydrophobic surfaces (cellulose nanofibrils (CNFs) and polyethylene terephthalate (PET), respectively). The effect of lipoxygenase treatment to minimize the affinity of the lipids with the given surface was considered. Application of an electroacoustic sensing technique (QCM) allowed the monitoring of the kinetics of oxidation as well as dynamics of lipid deposition on CNF and PET. The effect of the lipoxygenase enzymes (LOX) was elucidated with regards to their ability to reduce the formation of soiling lipid layers. The results pointed to the fact that the rate of colloidal oxidation depended on the type of lipid substrate. The pretreatment of the lipids with LOX reduced substantially their affinity to the surfaces, especially PET. Surface plasmon resonance (SPR) sensograms confirmed the effect of oxidation in decreasing the extent of deposition on the hydrophilic CNF. QCM energy dissipation analyses revealed the possible presence of a loosely adsorbed lipid layer on the PET surface. The morphology of the deposits accumulated on the solids was determined by atomic force microscopy and indicated important changes upon lipid treatment with LOX. The results highlighted the benefit of enzyme as a biobased treatment to reduce hydrophobic interactions, thus providing a viable solution to the control of lipid deposition from aqueous media.

INTRODUCTION

The adsorption properties of small organic molecules such as unsaturated fatty acids (UFAs) at solid–liquid interfaces are critical in order to modify the interfacial properties in a variety of fields, including paper-making, detergency, colloidal dispersions, wetting control, etc.1,2 Due to its commercial importance, great attention has been paid to the kinetics of adsorption of different fatty acids on ceramics, metal, and plastic surfaces.3–5 Many water-dispersible organic components are amphipathic, i.e., they comprise both hydrophobic and hydrophilic moieties; however, it is likely that chemical modification of these substances, for example, by oxidation, alters their adsorption behavior and their affinity with given surfaces. Among the different methods that have been suggested for oxidizing UFAs,6–9 lipoxygenases (LOXs) are proposed here as alternative oxidative enzymes. LOXs are a group of nonheme enzymes that are able to catalyze the dioxygenation of polyunsaturated fatty acids (PUFAs) consisting of at least one 1-cis-4 pentadiene system.10 LOXs have been found both in plants and animal tissues, where they participate in various biological roles.11–15 LOX main substrates include linoleic acid (C18:2) and arachidonic acid (C20:4).16–18 Among the LOXs that have been identified,19,20 LOX-1 is easily purified/isolated and is perhaps most relevant.21 Scheme 1 shows a simplified illustration of lipid oxidation induced by LOXs. In reactions with equimolar amounts of linoleic acid (LA), the ferric form of the enzyme catalyzes hydrogen abstraction from the bis-allylic (HC=CH-CH=CH2) carbon in the 11th position, stereospecifically, which results in the formation of a pentadienyl radical complex with the ferrous enzyme.20,22 Biomolecular oxygen (O2) reacts with the radical, to form a peroxy radical. This unstable peroxy radical oxidizes the iron to both reactivate the enzyme and form an anion. At the end, the hydroperoxy anion becomes protonated through the enzyme tunnel and produces the hydro-peroxide group on the lipid chain.23–25 Even though nonenzymatic oxidation may produce similar groups in lipids, it is unlikely that enzymatic lipid oxidation leads to a racemic mixture of hydroperoxides.26

There is a need to understand the interactions between lipids and polar/nonpolar components, upon LOX-induced oxidation, and to identify the role of developed hydrophilic groups on their affinity with exposed surfaces.27 Despite the interest that exists in relation to unsaturated fatty acids, it is not yet clearly understood how the addition of a hydrophilic group on a lipid chain, via LOX treatment, affects their adsorption properties. Here, we study the adsorption of two types of fatty
acids after LOX treatment, glycerol trilinoleate (GT) and LA, as well as isolated wood extractives. Two types of surfaces were considered as far as the extent and kinetics of adsorption of nonpolar components, namely, (1) hydrophobic polyester (polyethylene terephthalate, PET) and (2) hydrophilic cellulose nanofibers (CNF). These materials were chosen because of their relevance to washing, papermaking, and other processes where the surfaces are exposed to lipid colloids in aqueous media. The agglomeration of lipids in such cases may be unwanted since it can affect negatively production or performance.  

This is best exemplified by fouling (so-called deposits and stickies) of paper webs and processing fabrics, which translates into major losses of production. Wood extractives were also considered since they are often present in papermaking systems and owing to the fact that more than half of their composition comprises fatty acids and triglycerides. It is conceived that the adsorption of lipid colloids on the respective surfaces is primarily dominated by noncovalent interactions.

The dynamics of adsorption, as well as viscoelasticity of the adsorbed layer were investigated in situ and in real time by employing a quartz crystal microbalance with dissipation (QCM-D). The changes in lipid adsorption after oxidation were elucidated. For this purpose, a laminar-flow stream of aqueous media containing LOX-treated lipids was passed over the QCM sensor coated with the given film, and the adsorbed mass was monitored by recording the shift in resonance frequency. In a related context, the same technique has been used to study membrane surfaces relevant to water treatment. Additionally, surface plasmon resonance (SPR) was used to validate the QCM results together with atomic force microscopy (AFM), which was used to assess the morphology of the solids upon lipid attachment. The role of solution pH and buffer was also investigated in order to optimize the enzyme activity. This study introduces the benefits of LOX in order to reduce hydrophobic interactions and lipid deposition. PUFAs treated with isolated LOX are exposed to either hydrophilic or hydrophobic interfaces, in order to minimize surface accumulation.

**MATERIALS AND METHODS**

The reader is referred to the notes at the end of this Article for a list of abbreviations. Pure linoleic acid, GT, and soybean LOX from glycine (soybean) type I–B with 50 000 U/mg activity as well as boric acid and potassium hydroxide were obtained from Sigma-Aldrich and used as received. Hexafluoroisopropanol was purchased from Fisher Scientific. All the reagents and buffers were prepared using Milli-Q water (resistivity greater than 18 MΩ cm). Wood extractives were isolated from white pine wood chips using benzene-ethanol solvent according to TAPPI standard T 204 cm-97. PET was purchased from Scientific Polymer NY, USA and QCM sensors from Biolin Scientific, Sweden.

**LOX Solutions.** Ten milligrams of pure LOX enzymes was added to a 20 mL liquid scintillation vial under nitrogen gas, where the enzyme was dissolved in a 0.2 M borate and phosphate buffer separately with constant stirring at 200 rpm. Twenty milligrams of linoleic acid plus an equal amount of nonionic surfactant (Tween 20) was weighted into a 10 mL glass. Then the volume of the solution was made up to 10 mL with distilled water. Fresh substrate was used for each test. 0.3 mL of this solution was added to 2.7 mL of buffer with different pH, and finally 0.3 mL of LOX solution in the buffer was added to initiate the reaction. This solution was used for the subsequent enzyme activity tests that were determined by UV absorption, based on the formation of conjugated dienes, the concentration of which was detected as a linear change in absorbance at 234 nm wavelength (0.001 units per minute at 25°C–30°C).

**Ultrathin Films of CNF and PET.** Gold-coated QCM-D sensors were cleaned using a piranha solution (65% v/v H2SO4 + 30% v/v H2O2 (35%)) for 10 min, and then rinsed with milli-Q water, dried by nitrogen gas, followed by a UV/ozone cleaning for 15 min. The cleaned sensors were used for the subsequent thin film preparation. Thin films of PET were deposited on the sensors according to ref.

Briefly, a 0.15 wt % solution of PET in hexafluoroisopropanol was prepared using PET beads that were stirred for several hours until complete dissolution. Approximately 50–60 μL of this solution was placed on the surface of the cleaned gold-coated sensors and spun at 3000 rpm for 20 s under an infrared lamp (250 W) that was used to maintain the surface temperature at 85 °C (as monitored by an IR thermometer gun) before and during the spinning process.

Cellulose nanofibrils (CNFs) were produced by microfluidization of bleached and refined birch Kraft fibers. A sufficient amount of nanofibril gel was dispersed in milli-Q water to achieve a 1.67 g/L solids concentration. The fibrils were dispersed by using an ultrasonic micro tip homogenizer during 10 min at 25% amplitude (Branson Ultrasound Sonifier S-450, 400 W). The final dispersion was centrifuged for 30 min, and the supernatant was used for spin coating. Cleaned gold sensors were exposed to UV treatment for 15 min followed by immersion for 20 min in a 500 ppm solution of poly(ethyleneimine) (PEI), which was used as anchoring layer. The sensors were then rinsed with water and dried under nitrogen flow. Films were prepared by spin coating the fibril dispersion onto the PEI-treated sensor surface at 3000 rpm for 30 s. The sensors were placed in a desiccator until use.

**Treatment of Lipids and Wood Extractives with LOX.** Two types of pure oil substrates, GT and LA, as well as white-pine extractives (WE) were used for treatment with active LOX at room temperature. Experiments were carried out with dispersions in a stationary air atmosphere that were subjected to magnetic stirring (200 rpm) in the reaction vial. Ten milligrams of substrate was used in 10 mL of 0.2 M boric buffer (pH 9.0). In order to fully disperse the substrate, 10 min sonication was applied. Separately, an initial LOX stock solution was prepared, in which 10 mg of soybean LOX was dissolved in 10 mL 0.2 M boric buffer (pH 9.0) using a magnetic stirrer. One milliliter of this solution was added to the vial containing dispersed lipid and kept under stirring for 2 h at 200 rpm. At the end,
The dispersed solution was subject to another 10 min sonication for the purpose of evacuating the air bubbles from the solution. Thin Film Characterization. AFM imaging was performed to assess the morphology, roughness, and distribution of adsorbed lipids on the thin films of CNF and PET. AFM (Nano Scope III D3000 multimode scanning probe microscope, Digital Instruments, Inc.) was used in the noncontacting mode, in air. Scan sizes of 5 × 5 μm². Three different areas on each sample were analyzed. No image processing was employed except for flattening.

Dynamics of Lipid Adsorption. Adsorption and desorption phenomena on different surfaces were followed by either a Multi-Parametric Surface Plasmon Resonance (MP-SPR, SPR, Navi 200, Oy BioNavis Ltd., Tampere Finland) or a quartz crystal microbalance. The QCM and SPR techniques were used under similar conditions except for the flow rate, 100 versus 15 μL/min, respectively. In SPR, the changes in refractive index of the interface was used to calculate the adsorbed layer mass and its corresponding thickness. The change in light reflection angle due to the shift in the refractive index of the surface correlates with any change of mass, which alter the surface plasmon, layer mass and its corresponding thickness. The change in light (concentration, temperature, pH, rinsing protocol, etc.) except for the plateau signal in the sensograms, lipid solution was replaced with the solutions of known refractive indices. The QCM uses acoustic waves generated by an oscillating piezoelectric crystal plate to monitor mass adsorption or desorption. Applying an alternating electric current to the piezoelectric crystal quartz makes it oscillate at a certain frequency. Adsorption or desorption of any material on/from the crystal sensors can affect the frequency of the oscillation. The mass of the lipid layer was determined by measuring the change in frequency (in air oscillation) of the gold sensors before and after spin coating. The Sauerbrey equation was used as an approximation to calculate the relative mass that is adsorbed on the given surface.

\[ \Delta m = \frac{C \cdot \Delta f}{n} \] (3)

where \( \Delta m \) is the change in mass per unit area in ng/cm² or mg/m², \( \Delta f \) is the frequency change in Hz, \( C \) is the factor of sensitivity for the crystal (17.7 was used in this study), and \( n \) is the number of overtones. Therefore, the energy dissipation (D) is dependent on the frequency change (\( \Delta f \)) and the decay time (\( \tau \)) as follows:

\[ D = \frac{1}{\pi \Delta f \tau} \] (4)

where \( \tau \) values are determined by repeatedly disconnecting the oscillating crystals from the circuit via a computer-controlled relay.

RESULTS AND DISCUSSION

Enzyme Activity. Three distinct isozymes of LOX, namely, LOX-1, LOX-2, and LOX-3 have been described to be most effective in oxidizing fatty acids. It is reported that different pH optima and specific activity exist for these isozymes; while LOX-2 has the highest activity in pH 6.5, LOX-3 is active within a larger pH range (4.5–9.0). LOX-1 was used in this study for optimizing the enzyme activity. It was prepared in acidic, neutral, and alkaline colloidal solution using two types of buffers. As shown in Figure 1b, lower enzyme activity was seen for PBS, LOX showed a slightly better activity in BOR buffer. The exact reason is not well understood, but it can be due to the inhibitory effect of the buffer components that affect protein stability.

Surface Morphology upon Lipid Deposition. AFM imaging revealed lipid agglomeration on the tested surfaces and further indicated the role of substrate morphology on the lipid—lipid and lipid—substrate interactions. As can be observed...
in images of adsorbed WE (Figure 2, a1’-a2’), aggregates were mostly formed on PET. This observation agrees with the results obtained from QCM/SPR experiments to be discussed next and provides evidence of the lipid adsorption on polyester. The sizes of the lipid clusters were notably reduced when lipids were treated (oxidized) with LOX, particularly when PET was used as substrate. The PET surfaces had a higher roughness compared to those of CNF, and, at least to some limited extent, adsorption of the WE increased the roughness (Table 1). This may suggest that the agglomeration of hydrophobic components mostly took place on the upper layers of the film rather than areas underneath the surface. However, this phenomenon was less significant in the case of LOX-treated WE, given the fact that the oxidized lipids showed less affinity to polyester and the change in the film roughness was less notable. It was observed that the surface roughness of the CNF films followed similar behavior as that for PET; the deposition of lipids caused a slight increase in roughness. However, less agglomeration was observed on the cellulosic film compared to PET (Figure 2, b1’,b2’).

**Lipid Affinity with PET and CNF.** Generally, lipid components have a surface tension close to those of adhesives (~32 mJ/m²),50 and their affinity with surfaces tends to increase when the surface free energies are similar. This explains why wood extractives have a tendency to deposit onto hydrophobic surfaces. Using the condition that indicated the best LOX activity (pH, temperature and buffer type), similar systems were applied in heterogeneous phase, i.e., lipid adsorption on solid surfaces after enzyme treatment. The dynamics of adsorption of the model lipids as well as wood extractives are illustrated in Figure 3a,b for PET (hydrophobic) and CNF (hydrophilic) surfaces, respectively, under the same

**Table 1. Roughness values of PET and CNF thin films for AFM height images**

<table>
<thead>
<tr>
<th>PET/CNF film</th>
<th>roughness (nm)</th>
</tr>
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<tbody>
<tr>
<td>a-(PET)</td>
<td>66</td>
</tr>
<tr>
<td>a1-(PET-WE)</td>
<td>71</td>
</tr>
<tr>
<td>a2-(PET-WE-LOX)</td>
<td>68</td>
</tr>
<tr>
<td>b-(CNF)</td>
<td>6</td>
</tr>
<tr>
<td>b1-(CNF-WE)</td>
<td>11</td>
</tr>
<tr>
<td>b2-(CNF-WE-LOX)</td>
<td>8</td>
</tr>
</tbody>
</table>

*Figure 2.* Noncontact mode AFM images at 5×5 μm² for PET and CNF films: (a) PET; (b) CNF. a1 and b1 correspond to the surfaces after adsorption of unmodified WE. a2 and b2 correspond to adsorption of enzymatically treated WE. The panels indicated as a1’, b1’, a2’, b2’ are the corresponding phase images. The height and phase scale corresponds to z values as indicated by the height bar.
conditions. The negative shift in resonance frequency is associated with lipid adsorption on surfaces. The adsorption of the pure lipids and wood extractives decrease upon oxidative (LOX) treatment. Rinsing with buffer at time = 50 min was performed and continued until time = 60 min. Irreversible mass adsorption was observed in all cases, as indicated by the fact that the frequency recorded after rinsing did not return to the respective baseline. A faster adsorption process was observed on the PET surfaces, especially in the case of WE (Figure 3a). The QCM data show the irreversible adsorption of lipophilic components on both surfaces even after the enzymatic treatment, but, overall, the adsorption onto polyester-based films (PET) was higher and faster compared to that on the hydrophilic cellulose. The adsorption rate was significantly reduced following the oxidation process. In the case of CNF, the changes in the lipid affinity as a result of oxidation were more limited, especially for wood extractives adsorption. Interpretation of the results in the case of the cellulosic surfaces is more complex since they respond to the environment and are soft. Also, water coupling or hydration (changes in swelling and density) are expected.

**SPR Analysis.** The adsorption isotherms for the pure lipids and WE on PET and CNF surfaces, before and after LOX treatment were also acquired by SPR techniques, and results are summarized in Table 2. The SPR was used to validate the QCM results. By comparing the results of acoustic (QCM) and optical (SPR) methods, it was possible to monitor the changes in the film mass and thickness and to follow the kinetics of adsorption, lipid coverage, and molecular conformation. Compared to SPR, the QCM mass was overestimated for lipid adsorption, given the contribution of water coupling; however, the trends in the change of adsorbed mass onto CNF and PET calculated with both techniques were similar. It is apparent that pretreatment of lipids with the enzyme under an optimized condition (boric buffer, pH 9.0, 25 °C) reduce lipid deposition on PET surfaces.

**Lipid Layer Dissipation.** QCM ΔD−Δf curves (dissipation vs frequency profiles) for lipid adsorption on CNF and PET are included in Figure 4a,b. The analysis of energy dissipation provides additional information about the lipid adsorption.

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**Table 2. Lipids Mass Adsorption (mg/m²) from an Aqueous Media Measured by QCM and SPR on Thin Films**

<table>
<thead>
<tr>
<th>adsorbed mass (mg/m²) lipid</th>
<th>PET before-LOX</th>
<th>after-LOX</th>
<th>CNF before-LOX</th>
<th>after-LOX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before-LOX</td>
<td>after-LOX</td>
<td>before-LOX</td>
<td>after-LOX</td>
</tr>
<tr>
<td>LA</td>
<td>1.4</td>
<td>1.3</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>GT</td>
<td>4.3</td>
<td>2.9</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>WE</td>
<td>34.1</td>
<td>11.8</td>
<td>6.9</td>
<td>4</td>
</tr>
</tbody>
</table>

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Figure 3. Representative sensograms showing the shift of QCM frequency (third overtone) as a function of time upon adsorption of unmodified lipids: WE, LA and GT; after LOX-treated lipids: WE-LOX, LA-LOX, and GT-LOX. The adsorption occurs in aqueous media (1 mg/mL, pH 9.0, 25 °C) under constant flow rate of 100 μL/min. The surfaces correspond to (a,c) PET and (b,d) CNF.
behavior with or without oxidation. The measurement of changes in dissipation relates to the viscoelastic properties of the lipid layer. In general, compact and rigid adsorbed layers lead to relatively smaller changes in dissipation compared to that of a loosely attached layer. According to the results, for PET substrates, the energy dissipation for unmodified WE and GT are lower than LA. The large dissipation values for the untreated LA suggest a more extended attachment of the adsorbed lipid, and this behavior tends to continue even after the lipid was treated with LOX. However, the increase in slope of the dissipation profiles for GT and WE after LOX treatment (Figure 4a) was more noticeable upon adsorption on PET, indicating a different adsorption kinetics. This implies the more complex components (GT and WE) make a less rigid contact on PET after the oxidation, which probably is due to some repulsion between the lipid molecules and the PET surface. Furthermore, it seems the layers formed as a result of oxidized GT and WE were more viscoelastic compared to their corresponding unmodified lipids, which were more rigid.

The loss of energy dissipation for the oxidized lipids implies a loose attachment of the components to PET and can be due to the formation of new lipid structure as a result of enzyme oxidation: the addition of polar groups on the lipid chain may cause the lipid to be slightly more hydrophilic. This trend, however, was not seen for LA, which showed almost a linear relation in the profiles. Apparently, the configuration of the modified LA cause the same relative dissipation (a lower adsorbed mass and energy dissipation are recorded). From the same figure, one can conclude that oxidized GT and WE adsorb on PET as a more extended, viscoelastic layer.

Unmodified wood extractives adsorbed on PET to a large extent but caused little change in dissipation. Thus, a rigid layer was formed. However, a slight increase in the dissipation for lipids that were treated with the enzyme was observed, indicating that the oxidized components formed a looser adsorbed layer.

Tests with hydrophilic cellulose indicate that compared to the untreated lipids, most of the oxidized lipophilic components exhibited lower dissipation (Figure 4b). This is in agreement with the fact that the addition of polar groups to the fatty acids make them less hydrophobic and, as a result, the lipid adsorbed on CNF becomes more densely packed. Additionally, Figure 4b,d shows a difference in lipid adsorption on cellulose after enzymatic treatment, with a lower dissipation. Linoleic acid, which is a linear and small fatty acid, showed a different behavior, and the loss of dissipation was more significant for this fatty acid upon LOX oxidation. Probably, the modified LA, which is more hydrophilic, engages in hydrogen bonding with the primary alcohols on CNF and, as a result, the lipid adsorbs more densely. However, the dissipation decreased for WE and GT on CNF almost linearly with the amount adsorbed on the surface. The addition of polar groups to the lipid may increase hydration and increase the likelihood for removal of the lipid during washing or rinsing steps.

■ CONCLUSIONS

Experiments with electroacoustic and optical methods (QCM and SPR) suggested that lipids that were enzymatically modified with LOXs had a lower affinity to both lipophilic polyester and hydrophilic cellulose, owing to the changes in surface free energy. The alteration in the dissipation suggested a change in the hydration and viscoelasticity of the lipids that adsorbed on the surfaces. AFM images indicated limited lipid aggregation as well as changes in PET surface roughness upon enzymatic (LOX) treatment. The treatment with LOX of lipid colloidal Suspended in aqueous matrices inhibited their deposition of solid surfaces, signifying an environmentally safe method to control fouling in related processes.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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ABBREVIATIONS

PET poly(ethylene terephthalate)  
CNF cellulose nanofibrils  
PEI poly(ethylenimine)  
QCM quartz crystal microbalance  
SPR surface plasmon resonance  
LOX lipoxygenase  
BOR boric buffer  
PHS phosphate buffer  
WE white pine extractive  
WE-LOX extractives treated by lipoxygenase  
LA linoleic acid  
LA-LOX linoleic acid treated by lipoxygenase  
GT glycerol trilinolate  
GT-LOX glycerol trilinolate treated by lipoxygenase

REFERENCES

(35) Conterras, A.; Steiner, Z.; Miao, J.; Kascher, R.; Li, Q. Studying the Role of Common Membrane Surface Functionalities on...


