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Comparative evaluation of manganese peroxidase- and Mn(III)-initiated peroxidation of C18 unsaturated fatty acids by different methods

Alexander N. Kapich[a,b,c,*], Tatyana V. Korneichik[c], Kenneth E. Hammel[a], Annele Hatakka[b]

[a] Institute for Microbial and Biochemical Sciences, USDA Forest Products Laboratory, One Gifford Pinchot Dr., Madison, WI 53726, USA
[b] Department of Food and Environmental Sciences, University of Helsinki, Viikinkaari 9, Viikki Biocenter 1, P.O. Box 56, 00014 Helsinki, Finland
[c] International Sakharov Environmental University, 23 Dolgobrodskaya Str, 220009 Minsk, Belarus

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ABSTRACT

The peroxidation of C18 unsaturated fatty acids by fungal manganese peroxidase (MnP)/Mn(II) and by chelated Mn(III) was studied with application of three different methods: by monitoring oxygen consumption, by measuring conjugated dienes and by thiobarbituric acid-reactive substances (TBARS) formation. All tested polyunsaturated fatty acids (PUFAs) were oxidized by MnP in the presence of Mn(II) ions but the rate of their oxidation was not directly related to degree of their unsaturation. As it has been shown by monitoring oxygen consumption and conjugated dienes formation the linoleic acid was the most easily oxidizable fatty acid for MnP/Mn(II) and chelated Mn(III). However, when the lipid peroxidation (LPO) activity was monitored by TBARS formation the linolenic acid gave the highest results. High accumulation of TBARS was also recorded during peroxidation of linoleic acid initiated by MnP/Mn(II). Action of Mn(III)-tartrate on the PUFAs mimics action of MnP in the presence of Mn(II) indicating that Mn(III) ions are involved in LPO initiation. Although in our experiments Mn(III) tartrate gave faster than MnP/Mn(II) initial oxidation of the unsaturated fatty acids with consumption of O_2 and formation of conjugated dienes the process was not productive and did not support further development of LPO. The higher effectiveness of MnP/Mn(II)-initiated LPO system depends on the turnover of manganese provided by MnP. It is proposed that the oxygen consumption assay is the best express method for evaluation of MnP- and Mn(III)-initiated peroxidation of C18 unsaturated fatty acids.

1. Introduction

Manganese peroxidase (MnP) is the most common lignin-modifying enzyme produced by almost all white-rot wood-decaying basidiomycetous fungi [1,2]. This enzyme is considered to play a crucial role in lignin degradation [3–6]. MnP is an extracellular heme containing peroxidase that requires Mn(II) as its reducing substrate. The enzyme oxidizes Mn(II) to Mn(III), which is chelated by organic acids such as oxalate or malonate. Mn(III)-chelator complexes in turn are efficient oxidants and act as low-molecular weight, diffusible redox-mediators. Thus, MnP oxidizes phenolic lignin substructures in wood via diffusible Mn(III)-chelator complexes formed during peroxidase cycle of the enzyme. However, non-phenolic units that predominate in natural lignin are not susceptible to degradation by this mechanism.

The MnP can also oxidize unsaturated fatty acids possibly through Mn(III) chelates thereby generating lipid peroxyl and other lipid radicals, which in turn can be the primary agents of fungal attack on non-phenolic lignin in wood [7–9]. It means that lipid peroxidation (LPO) may be involved in lignin degradation by the white-rot wood-decaying fungi. The same chemistry may be involved in the degradation of recalcitrant xenobiotics by the fungi [10,11].

The environmental importance of LPO initiated by MnP explains the interest to the methods that may be applied to follow LPO in this system. It is generally recognized that the nature and amount of LPO products may considerably vary depending on the system studied, the type of pro-oxidants and many other factors [12]. This problem is complicated by the fact that individual fatty acids may produce different products of LPO which give different results with different methods of LPO evaluation [13]. Because of the complexity of the LPO pathways and because many factors affect the amounts of the products formed, it is recommended to measure two or more indexes of LPO simultaneously [14].

Earlier, we have already proposed a rapid method to quantify the activity of LPO in MnP-initiated systems and in cultures of wood-decaying fungi that gives total evaluation of the activity of LPO

* Corresponding author at: International Sakharov Environmental University, 23 Dolgobrodskaya Str, 220009 Minsk, Belarus. Tel.: +375 17 2302678; fax: +375 17 2306897.
E-mail address: ankapich@mail.ru (A.N. Kapich).

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by oxygen consumption rate [15]. The determination of the conjugated dienes and substances reacting with thiobarbituric acid (TBARS) gives an option to control primary and secondary products of LPO. The goal of this research was to compare the methods for evaluation of the activity of LPO of C18 unsaturated fatty acids by MnP/Mn(II) and Mn(III)-chelator complexes as well as to obtain additional information regarding mechanisms of MnP- and Mn(III)-initiated LPO.

2. Materials and methods

2.1. Reagents and enzymes

Low carbonyl and peroxide 10% Tween 20 solutions were purchased from Thermo Fisher Scientific Inc., Mn(III) acetate and 2-thiobarbituric acid (TBA) from Sigma–Aldrich Co., oleic (18:1), linoleic (18:2) and linolenic (18:3) acids from Nu-Chek-Prep Inc. Mn(III) tartrate solutions were prepared by dissolving Mn(III) acetate in 100 mM sodium tartrate (pH 4.5) in darkness immediately prior to use [16].

Two MnP preparations were used throughout this study. MnP isoenzyme P5 was purified by Mono-Q- anion-exchange chromatography from the extracellular culture fluids of *Phanerochaete chrysosporium* ME-446 grown on wheat straw-containing GPCSL medium as described [17]. Recombinant *P. chrysosporium* MnP (H4 isoenzyme) was expressed in cultures of *Aspergillus oryzae* and was purified by anion-exchange chromatography on DEAE-Biogel A as described [18]. MnP activity was determined spectrophotometrically (Shimadzu PharmaSpec UV-1700 or Shimadzu MultiSpec-1501, Japan) by following the formation of 2,6-dimethoxyphenol (DMP) dimer (diphenoquinone) at 469 nm (molar extinction coefficient = 49600 M^{-1} cm^{-1}) [16]. One unit (U) of MnP activity represents 1 μmol of the diphenoquinone formed in 1 min at room temperature. The MnP isoenzyme preparations were also tested for oxidation of veratryl alcohol to veratraldehyde at 310 nm to confirm that there is no lignin peroxidase activities present [19].

2.2. Oxygen consumption experiments

The oxygen consumption during MnP and Mn(III)-initiated peroxidation of C18 fatty acids was monitored at 25 °C with the Hansatech Instruments Oxygraph (England, UK) [15]. The maximal rates of oxygen consumption were taken from the linear portions of Oxygraph recordings and were calculated using the Oxygraph software. The procedure included consecutive addition of the components to the reaction chamber containing the oxygen electrode. The reaction mixtures for MnP-initiated LPO (1 ml) contained 50 mM sodium acetate buffer (pH 4.5), 1 mM emulsion of unsaturated fatty acid, 0.1% (wt/vol) Tween 20, and 1 mM $MnSO_4$. The fatty acids were emulsified beforehand in argon saturated Tween 20 (100 mM of the corresponding fatty acid in 10% Tween 20). The reactions were initiated by adding 0.015 U of purified MnP P5 isoenzyme preparation. The reaction mixtures for Mn(III) tartrate-initiated LPO contained the same buffer and fatty acid emulsions but MnP and $MnSO_4$ were not included. Instead, these reactions were initiated by adding 0,04 mM Mn(III) tartrate. In some experiments 0.2 mM and 0.02 mM of Mn(III) tartrate were used to initiate LPO. Every oxygen consumption experiment was repeated at least three times with basically identical outcomes.

2.3. Conjugated dienes formation

The kinetics of MnP and Mn(III)-initiated formation of conjugated dienes was assayed spectrophotometrically by monitoring the increase in absorption at 233 nm. The reactions were carried out directly in 1-cm quartz cuvette as described [9]. The reaction mixtures for both systems contained the same components as it was used in oxygen consumption experiments but MnP H4 isoenzyme was used in these experiments.

2.4. Determination of thiobarbituric acid-reactive substances (TBARS)

The accumulation of TBARS was followed during peroxidation of C18 unsaturated fatty acids initiated by MnP/Mn(II) or Mn(III) tartrate. The reaction mixtures for MnP-initiated reactions (1 ml) contained 20 mM sodium acetate buffer (pH 4.5), 1 mM $MnSO_4$, and 5 mM of the corresponding fatty acid that had been emulsified in argon-saturated Tween 20 (1% wt/vol final concentration). The reactions were initiated by adding 0.3 U of MnP H4 isoenzyme. The reaction mixtures for Mn(III)-initiated lipid peroxidation contained the same buffer and fatty acid emulsion as above without MnP and $MnSO_4$. Instead, these reactions were initiated by adding 0.2 mM Mn(III) tartrate. Controls without MnP and Mn(III) tartrate were set up for each variant. The assay mixtures for TBARS contained 150 μl of the reaction mixtures, 200 μl of 6% sodium lauryl sulfate, 1.5 ml of 2% phosphoric acid, 0.5 ml of thiobarbituric acid solution (0.8% wt/vol) and 50 μl of 10 mM butylated hydroxytoluene in ethanol. The thiobarbituric acid solution was prepared as described [20]. The mixtures were capped to prevent evaporation and incubated at 100 °C for 15 min. The absorbance of the cooled assay mixtures was measured at 532 nm.

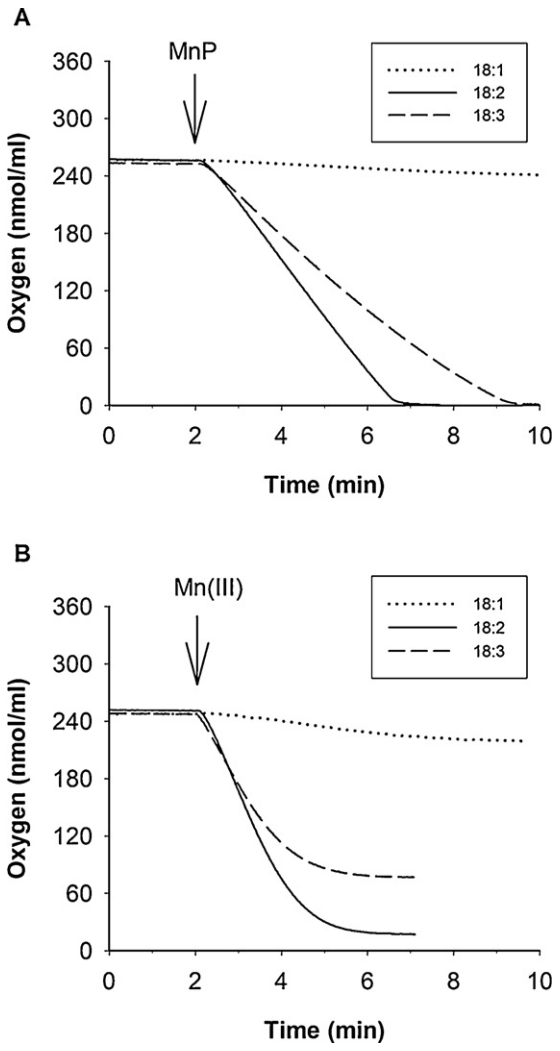

Fig. 1. Oxygen consumption during reactions of MnP- (A) and Mn(III) (B) -initiated peroxidation of C18 unsaturated fatty acids (18:1, 18:2 and 18:3). Reaction mixtures contained: (A) 50 mM sodium acetate buffer (pH 4.5); 1 mM unsaturated fatty acid; 0.1% Tween 20; 1 mM $MnSO_4$; 0.015 U of purified MnP P5. (B) 50 mM sodium acetate buffer (pH 4.5); 1 mM unsaturated fatty acid; 0.1% Tween 20; 0.04 mM Mn(III) tartrate. Arrows indicate the addition of MnP P5 and Mn(III) tartrate.

3. Results

3.1. Evaluation of MnP/Mn(II) and Mn(III)-initiated LPO by oxygen consumption assay

As it was determined by the oxygen consumption assay, the purified MnP isoenzyme P5 of *P. chrysosporium* was able to oxidize all tested C18 unsaturated fatty acids in the presence of Mn(II) ions (Fig. 1A). The oxidation of polyunsaturated fatty acids (linoleic and linolenic acids) continued till complete oxygen consumption from the reaction mixtures. Linoleic acid was oxidized with the highest speed of O_2 consumption that reached 60.4 nmol ml^{-1} min^{-1}. More unsaturated linolenic acid that has three double bonds was oxidized slower than less unsaturated linoleic acid that has only two double bonds. Monounsaturated oleic acid that has only one double bond was also oxidized although very slowly in the system as seen by O_2 consumption test.

Since Mn(III) chelates are considered to be the proximal oxidants in the systems with MnP-initiated lipid peroxidation, we next assessed the oxygen consumption in the reactions of Mn(III) tartrate (0.04 mM) with the C18 unsaturated fatty acids. Fig. 1B shows

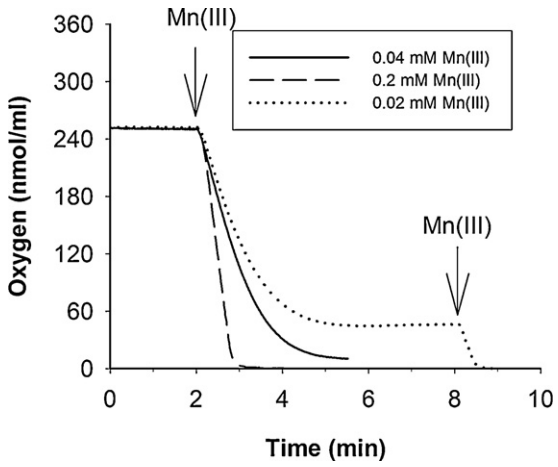

Fig. 2. Effect of Mn(III) tartrate concentration on the rate of oxygen consumption in the reactions of Mn(III)-initiated peroxidation of linoleic acid. Reaction mixtures contained: 50 mM sodium acetate buffer (pH 4.5); 1 mM linoleic acid; 0.1% Tween 20; 0.02, 0.04 or 0.2 mM Mn(III) tartrate. Arrows indicate the addition of Mn(III) tartrate.

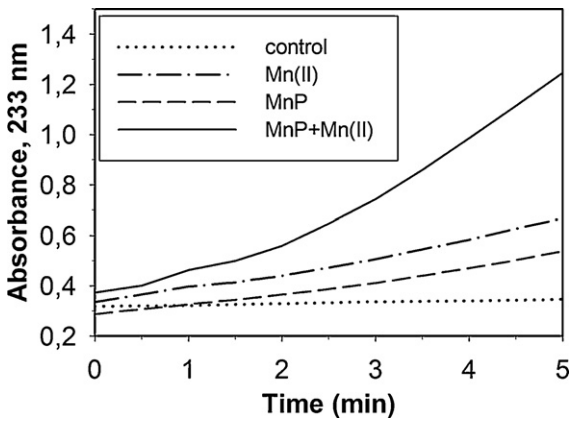

Fig. 3. Time course of conjugated dienes formation during peroxidation of linoleic acid in the absence of MnP and Mn(II) (control) or in the presence of Mn(II) or MnP or both. Complete reaction mixture (MnP + Mn(II)) contained: 50 mM sodium acetate buffer (pH 4.5); 1 mM unsaturated fatty acid; 0.1% Tween 20; 1 mM $MnSO_4$; 0.015 U of purified MnP H4.

that Mn(III) tartrate initiated peroxidation of the C18 polyunsaturated fatty acids much more actively than MnP/Mn(II) system. However, the order of the oxidizability of the fatty acids in this case was the same as that shown by MnP in the presence of Mn(II) – the linoleic acid was oxidized by Mn(III) tartrate with the highest rate of O_2 consumption that reached 170 nmol ml^{-1} min^{-1} in this case. Oleic acid was also oxidized by Mn(III) although with very low rate of oxygen consumption.

It is noteworthy that oxygen was not consumed completely in all reactions where LPO was initiated by 0.04 mM of Mn (III) tartrate. Even in the case of the oxidation of linoleic acid the dissolved O_2 was not consumed completely (Fig. 1B). After very fast consumption of O_2 in the beginning the reaction stopped after several minutes. This effect has been even better seen when linolenic acid was oxidized by Mn(III) tartrate. The linolenic acid was oxidized not only slower than linoleic acid but in addition the consumption of oxygen stopped after 6 min of the reaction when O_2 concentration was still higher than 60 nmol ml^{-1}.

Increase of the concentration of Mn(III) tartrate to 0.2 mM resulted in complete and much faster consumption of the dissolved O_2 in the reaction with linoleic acid (Fig. 2). On the contrary, decrease of the Mn(III) tartrate concentration to 0.02 mM reduced the O_2 consumption and it stopped at the level of about 45 nmol ml^{-1}. Addition of Mn (III) tartrate after 8 min in this case initiated peroxidation of the linoleic acid again and all O_2 was finally consumed. This result shows that not complete oxygen consumption in the Mn(III)-initiated linoleic acid peroxidation was due to disappearance of Mn(III) from the reaction mixture.

3.2. Evaluation of MnP/Mn(II) and Mn(III)-initiated LPO by conjugated dienes formation

The UV spectral changes observed during the reaction of MnP with PUFAs clearly indicated formation of conjugated dienes which is seen as the increase of the absorption peak at 232–234 nm. Fig. 3 shows the production of the conjugated dienes during oxidation of linoleic acid in the presence and in the absence of MnP and Mn(II) ions. It is noteworthy that there is a problem with this test because polyunsaturated fatty acids can be slowly oxidized themselves by the spectrophotometer source of UV light during monitoring of the increase of the absorption at the UV area of the spectrum. Moreover, addition of Mn(II) or MnP to the emulsion of linoleic acid increased the rate of the oxidation indicating to their photosensitizing properties. However, complete enzymatic system including both MnP and Mn(II) provided about 3 times higher rate of conjugated dienes production than in the cases when they were added separately.

To compare the activity of peroxidation via conjugated dienes production we reproduced the experiments with the enzymatic MnP/Mn(II) system using different C18 unsaturated fatty acids according to [9]. The obtained results confirmed that the dienoic linoleic acid was oxidized at the highest rate in the system. The rate of conjugated dienes formation clearly slowed down for linolenic acid. The conjugated dienes were not formed during the reaction with monoenoic oleic acid because it contains only one double bond and therefore is not able to produce conjugated dienes. Formation of conjugated dienes was also maximal and very fast during Mn(III)-initiated peroxidation of linoleic acid.

3.3. Evaluation of MnP/Mn(II) and Mn(III)-initiated LPO by TBARS production

During MnP/Mn(II)-initiated LPO TBARS production was maximal for linolenic acid (Fig. 4A). It started without any lag phase and reached maximum concentration of TBARS after 180 min. In case of the oxidation of linoleic acid TBARS formation had a short lag phase (about 10 min) and reached its maximum after 120 min. The level of TBARS in the reaction with linoleic acid was 2 times lower than in case with linolenic acid. No TBARS formation was found during oxidation of oleic acid by MnP.

As seen from Fig. 4B, there was also no accumulation of the secondary aldehyde TBARS products in the Mn(III) tartrate-dependent system with oleic acid and very low TBARS accumulation in case of the oxidation of linoleic acid. Relatively higher accumulation of TBARS was found in case of the oxidation of linolenic acid. However, in this case TBARS reached its maximum after 10 min of the reaction and then slowly decreased until 240 min. This shows that the process of LPO was not further developed in case of initiation by chelated Mn(III) and it stopped soon after its initiation. In contrast, the process of linolenic and linoleic acid peroxidation in the system with MnP/Mn(II) was productive and resulted to much higher accumulation of the secondary TBARS products (Fig. 4A).

3.4. Reduction of Mn(III) to Mn(II) during oxidation of C18 unsaturated fatty acids

Formation of conjugated dienes during Mn(III)-initiated peroxidation of linoleic acid seen as an increase in the absorbance at 233 nm was accompanied by the reduction of Mn(III) tartrate seen

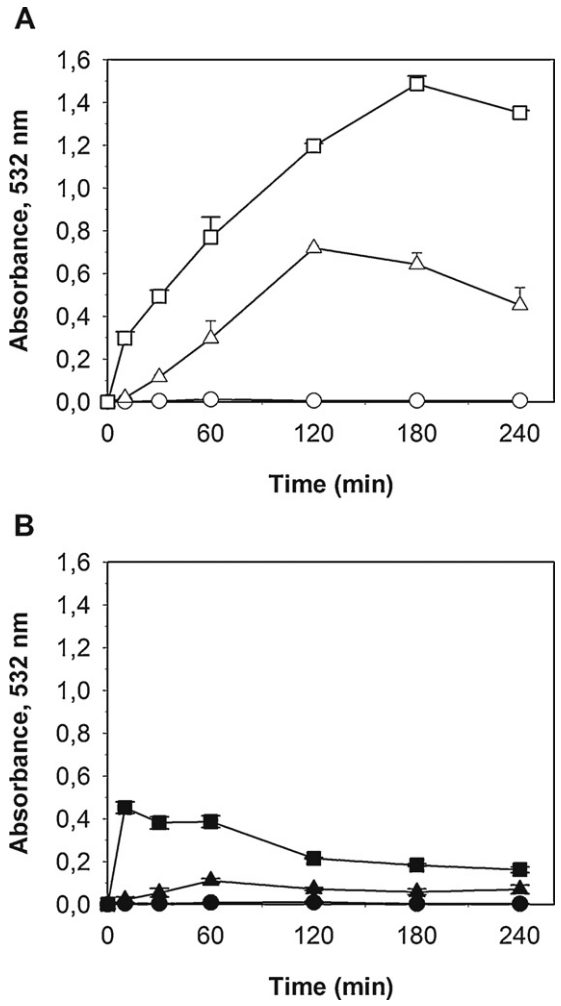

Fig. 4. Time course of TBARS formation during peroxidation of C18 unsaturated fatty acids: C18:1 (circles), C18:2 (triangles) and C18:3 (squares) initiated by MnP/Mn(II) (A) or Mn(III) tartrate (B). Reaction mixtures contained: (A) 20 mM sodium acetate buffer (pH 4.5); 5 mM unsaturated fatty acid; 1% Tween 20; 1 mM $MnSO_4$; 0.015 U of purified MnP H4. (B) 20 mM sodium acetate buffer (pH 4.5); 5 mM unsaturated fatty acid; 1% Tween 20; 0.2 mM Mn(III) tartrate.

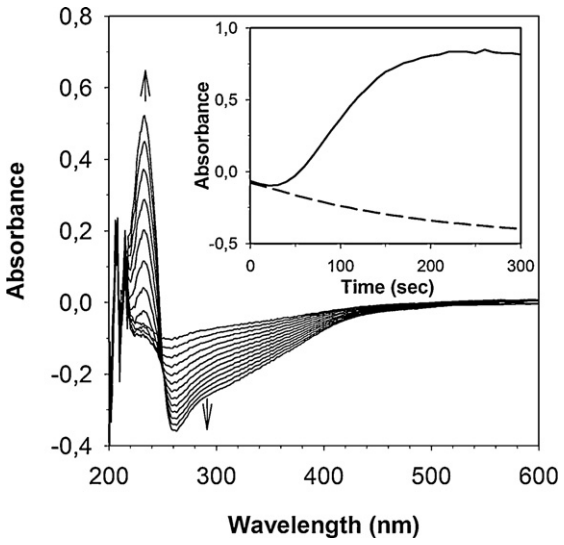

Fig. 5. UV absorption spectra changes during Mn(III)-initiated peroxidation of linoleic acid. Reaction mixtures contained 50 mM sodium acetate buffer (pH 4.5); 0.2 mM linoleic acid; 0.04% (wt/vol) Tween 20; 0.2 mM Mn(III) tartrate. Spectra were taken at 30 s intervals. *Inset*: The increase of absorption at 233 nm (solid line) shows the formation of conjugated dienes. The decrease of absorption at 290 nm (dashed line) shows the reduction of Mn(III).

as the decrease of absorption at 290 nm (Fig. 5). These spectral changes resulting from incubation of Mn(III) tartrate with emulsion of linoleic acid in 10% Tween 20 were well observed when low concentration of linoleic acid (0.2 mM) was applied. The linoleic acid hydroperoxides with conjugated diene structures had a UV spectrum centered on 233 nm, with no contribution above 250 nm. We monitored the Mn(III) tartrate reduction at 290 nm as it was proposed before [16]. The reduction of Mn(III) started immediately after its addition to the emulsion of linoleic acid and continued even after maximum of the conjugated dienes was reached. Solutions of Mn(III) tartrate lost their absorbance at 290 nm and became colorless due to reduction of Mn(III) to Mn(II) even in the absence of the unsaturated fatty acids but addition of the linoleic acid stimulated the process (data not shown). No precipitates of Mn(IY) oxides were formed in these reactions that confirms that the disappearance of Mn(III) was due to reduction of Mn(III) to Mn(II).

4. Discussion

In this study we show that diene and triene C18 PUFAs may be oxidized by MnP in the presence of Mn(II) ions and that the MnP-initiated LPO of the PUFAs may be monitored by oxygen consumption assay, by detection of conjugated dienes and by determination of TBARS. Oxygen consumption during this process results mainly from reactions between carbon-centered lipid radicals and molecular oxygen and may be overall indication of the activity of the process [15,20]. The conjugated dienes appear through the conversion of the PUFAs to PUFA-hydroperoxides that have UV absorption maximum at 232–234 nm [21,22]. PUFA-hydroperoxides with conjugated double bonds may be considered as primary products of LPO and the rate of their formation shows initial speed of the process. TBARS are secondary oxidation products of the LPO including malonaldehyde and other related compounds formed when lipid hydroperoxides break down. In these terms accumulation of TBARS shows completeness of the process of LPO [25,26].

It is well-known that susceptibility of unsaturated fatty acids to oxidation in homogeneous systems increases with the number of double bounds in their molecules and it is linearly dependent on the number of *bis*-allylic methylenes in the fatty acids [23–25]. That is why linolenic acid (18:3) containing three double bounds is more easily oxidizable fatty acid than containing two double bounds linoleic acid (18:2). However, the comparison of the activity of peroxidation of C18 unsaturated fatty acids determined by O_2 assay and by conjugated dienes formation shows that the rate of MnP- and Mn(III)-initiated peroxidation was maximal for linoleic acid. These results suggest that the linoleic acid was the best substrate for LPO in the systems. Some other studies with oil in water emulsions have also found that in aqueous micellar solutions less unsaturated fatty acids, in particular linoleic acid, are paradoxically more prone to LPO than more unsaturated fatty acids [13,26,27]. It may be explained by the hypothesis suggesting that peroxyl radicals of linolenic acid are more polar than linoleic acid peroxyl radicals that results in their migration to the periphery of the micelles, where they cannot participate efficiently in radical chain propagation developing LPO.

Opposite result was obtained when we monitored activity of LPO by TBARS formation. Higher level of TBARS accumulation was found in case of MnP- and Mn(III)-initiated peroxidation of linolenic acid. It is generally accepted that malondialdehyde (MDA) appears to be the major aldehyde that reacts with TBA forming the colored complex [21,22]. MDA mainly results from the oxida-

tive degradation of polyunsaturated fatty acids with more than two methylene-interrupted double bonds. From studied unsaturated C18 acids only linolenic acid could be precursor for MDA that explains the highest extent of TBARS accumulation in this case. However, this fact does not mean that the rate of linolenic acid peroxidation was higher than the rate for linoleic acid.

It is generally agreed that oleic and linoleic acids are weak precursors for MDA [28,29]. Moreover, in many studies the MDA was not found during peroxidation of oleic acid [21,22]. The yield of MDA from peroxidation of linoleic acid was found to be about 10 times less than from other more unsaturated fatty acids [29]. Instead, some other aldehydes, in particular 2,4-decadienal, 4-hydroxy-2-nonenal, hexanal, glyoxal and several others are produced during peroxidation of linoleic acid [21,30]. The production of some of the aldehydes has been shown in the MnP-initiated linoleic acid peroxidation systems [31,32]. However, the extent of contribution of these aldehydes to the red pigment formation in the TBA test is low [33]. That is why the TBA test underestimates the oxidation products resulting from peroxidation of linoleic acid [21].

Nevertheless, in our experiments with MnP-initiated peroxidation of linoleic acid TBARS accumulation was quite high and only two times lower than that with linolenic acid. It indicates that the accumulation of the TBA reactive aldehydes derived from linoleic acid and other than MDA was high so as the rate of peroxidation of the acid. This assumption is in agreement with our data that have been received by oxygen consumption assay and by conjugated diene test showing that linoleic acid is oxidized by MnP and Mn(III) with the highest rate in comparison to other C18 unsaturated fatty acids.

This research gives also additional information regarding mechanisms of MnP- and Mn(III)-initiated LPO. It seems that the cessation of the LPO in the system with Mn(III) tartrate is caused by very fast reduction of the pro-oxidant – Mn(III) to antioxidant – Mn(II) as shown by monitoring the absorbance of the Mn(III) tartrate complex (Fig. 5). The chain-breaking antioxidant activity of Mn(II) is well-documented [34,35]. In the enzymatic MnP/Mn(II)-driven system the constant recycling of manganese was provided with oxidation of Mn(II) to Mn(III) by MnP and reduction of Mn(III) to Mn(II) in the course of linoleic acid peroxidation. It seems that the recycling of manganese in the MnP-catalyzed reactions is the driving force for LPO of C18 unsaturated fatty acids and as a result for non-phenolic lignin degradation.

In conclusion, evaluating adequacy of the tested methods for LPO measurements of the C18 unsaturated fatty acids in the MnP- and Mn(III)-initiated systems we have to stress that each method has its own drawbacks and advantages but the oxygen consumption assay is the best express method for comparative studies that gives valuable information about total activity of LPO in the systems.

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