Inhibition of Linoleic Acid Oxidation by Interaction with a Protein-Rich Oat Fraction

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A protein-rich fraction from oat was found to protect linoleic acid against oxidation in an aqueous suspension containing soybean lipoxygenase-1 and micellar linoleic acid. In this system the oat fraction reduced the initial oxidation rate of linoleic acid by 50% when the oat fraction/linoleic acid ratio was 5:1 (w/w). The oat fraction did not act on the lipoxygenase enzyme but reduced the concentration of linoleic acid that serves as a substrate for lipoxygenase-1. To achieve the reduction in the oxidation rate a contact between linoleic acid and the oat fraction was required. The efficiency of the protection was dependent on the duration of this contact: the maximum protection was reached after a 5-min incubation period. However, total cessation of oxidation was not reached with any concentration of the oat fraction, indicating that the oxidizible and non-oxidizible forms of linoleic acid are in equilibrium. Because lipoxygenase-1 prefers the monomeric form of the substrate, the present findings agree with the hypothesis that the oat fraction reduces the concentration of monomolecular form of substrate. In most food systems monomolecular free linoleic acid is liberated slowly and at relatively low concentrations, therefore, even a small amount of the oat fraction would guard the system from oxidative deterioration.

Keywords: *Oat; lipid stability; monomolecular linoleic acid; oxidation*

INTRODUCTION

In pure oils the oxidation rate of lipids can be predicted to some extent by some key factors including the degree of fatty acid unsaturation, distribution of fatty acids into different lipid classes, temperature, and the presence of oxygen, antioxidants, and oxidation catalysts. However, when lipids are present as part of a complicated food system, the factors mentioned above can be overridden by the factors arising from the physical coordination of lipids and other components in the food matrix (Frankel, 1999; Fritsch, 1994). In such a system, lipids are distributed into different phases such as membranous structures, liposomes, or micelles, or complexed with different host molecules. At any given time, a proportion of lipids is also found as premicellar aggregates or free in the bulk water. The two latter forms are in equilibrium with micelles and are radically more susceptible toward oxidation reactions than the other forms (Lagocki et al., 1976).

In the literature, the lipids in dough and in aqueous suspensions of cereals are grouped into bound and unbound pools. Although this classification is based on the extractability, the oxidation rate of lipids in the bound pool differs greatly from that in the unbound pool (Mann and Morrison, 1974). Upon mixing the dough, the proportion of bound lipids increases rapidly while the rate of lipid oxidation concurrently falls dramatically (Delcros et al., 1998; Chung and Tsen, 1975; Graveland, 1970). By inducing changes in the physical coordination of lipids toward forms that are less susceptible to lipid deterioration, better control over the lipid deterioration during cereal processing could be gained without extensive inactivation of the lipolytic enzymes or addition of antioxidants.

The present study describes the kinetic behavior of lipid oxidation in aqueous suspensions containing the oat (*Avena sativa*) fiber fraction (FABOF) previously found to be an effective inhibitor of lipid oxidation (Lehtinen and Laakso, 1998). Among the major cereals, oat differs in its high fat content and high lipolytic enzyme activity (Youngs, 1978). Although these factors make oat-based products liable to lipid deterioration, oat carries also a high capacity to retard oxidation in the form of both the phenolic antioxidants (Dimberg et al., 1993; Tian and White, 1994; Emmons and Peterson, 1999) and the high capability to bind lipids (Lehtinen and Laakso, 1997; Ma, 1984).

MATERIALS AND METHODS

Preparation of the Fatty-Acid-Binding Oat Fraction. The preparation of FABOF with enhanced capability to inhibit lipid oxidation was carried out essentially as described by Lehtinen and Laakso (1998). The industrially made oat fiber was re-milled (Fritsch, Pulverizette 14, sieve ring 0.5 mm) to give finer flour. FABOF was obtained by washing the fiber with slightly acid buffer solution and subsequently extracting the fiber with alkaline solution. To collect FABOF, the pH of the alkaline extract was adjusted to pH 6 and the precipitate formed was lyophilized to yield FABOF.

Measurement of Linoleic Acid Distribution by Lipoxygenase Assay. When oat is mixed in water, a heterogeneous system where free fatty acids are distributed among several pools with different microenvironments is obtained. The observed rate of lipoxygenase reaction in such a system is dependent on the concentration of linoleic acid in the free monomer pool. Under proper conditions of lipoxygenase assay any change that alters the distribution of linoleic acid between

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different pools can be observed as a change in the reaction rate (Galpin and Allen, 1977). On the basis of this principle, the oxygraphic lipoxygenase assay was used to monitor possible changes that FABOF could cause in the concentration of monomer linoleic acid.

The rate of linoleic acid oxidation was measured by following the rate of oxygen consumption of aqueous suspensions by polarography (Clark electrode, YSI 5300). Varying amounts of FABOF, 0.86 mg of linoleic acid (Sigma L-1376, St. Louis, MO) in 0.3 mL of Tween-20/NaOH (Axelrod et al., 1981) and 0.2 M sodium phosphate buffer pH 7.0 to make the total volume 7.3 mL were incubated for different periods of time in a reaction cuvette thermostated to 25 °C. After the preincubation period, soybean lipoxygenase (Sigma L-8383, St. Louis, MO, 0.1 mg/mL in 0.05 M sodium phosphate buffer pH 8.0) was added to the solution. The rate of oxygen consumption was recorded. A similar experiment, where FABOF was preincubated with the lipoxygenase solution and the reaction was initiated with linoleic acid substrate, was also carried out.

Tween-20 solutions similar to that described for linoleic acid substrate (Axelrod et al., 1981) were also made from palmitoleic and oleic acids (Sigma O-1008, P-9147, St Louis, MO). To study the effect of these fatty acids on the capability of FABOF to inhibit linoleic acid oxidation, 4.4 mg of FABOF in 0.2 M sodium phosphate buffer pH 7.0, was preincubated 5 min with 0.25−0.50 mg of these acids prior to addition of 0.86 mg linoleic acid. The solution was incubated for a further 5 min and the reaction was initiated with the addition of the lipoxygenase.

Determination of Fatty Acid Distribution into Different Lipid Classes. To elucidate whether the supplemented fatty acids were acylated into glycerol structures, 10 mg of FABOF was incubated at 25 °C with 1.5 or 3.0 mg of linoleic acid (Sigma L-1376, St. Louis, MO) in Tween-20/NaOH (Axelrod et al., 1981) and 0.2 M sodium phosphate buffer pH 7.0 to make the total volume 7.3 mL. After the 15-min incubation the suspension was freeze-dried and extracted twice (2h and 2h) at 25 °C with 20 vol chloroform/methanol (2:1) solution.

Absolute amounts of different fatty acids in triglycerides, diglycerides, free fatty acids, and polar lipids were determined after TLC-separation as described by Liukkonen et al. (1992). The distribution of linoleic acid in the different lipid classes, as well as that in the extraction residue, was determined by converting the fatty acids to methyl esters essentially as described by Suutari et al. (1990). Methyl esters were identified and quantitated by gas chromatography with a flame ionization detector. At least one set of duplicated analyses was performed.

RESULTS AND DISCUSSION

The addition of FABOF to the lipoxygenase assay system, in which linoleic acid is mainly in the micellar form, decreased the linoleic acid oxidation rate markedly, indicating that the concentration of linoleic acid in the free monomeric pool was reduced (Figure 1). Because of the heterogeneous composition of FABOF and the micellar nature of linoleic acid substrate, no stoichiometric data can be obtained from the direct interactions between the monomer linoleic acid and FABOF. However, some practical estimations can be drawn about the quantities of FABOF needed to decrease the oxidation rate of free fatty acids formed, e.g., during food processing. For example, an extensive hydrolysis of lipids in barley and oat flours yields 15−40 mg of free fatty acids per one gram of flour (Liukkonen et al., 1992). On the other hand, on the basis of the data shown in Figure 1, a 50% reduction in the lipoxygenase reaction rate of such an amount of free linoleic acid could be achieved by adding 75−200 mg of FABOF per 1 g of flour. For the subsequent experiments, the FABOF concentration was chosen so that the

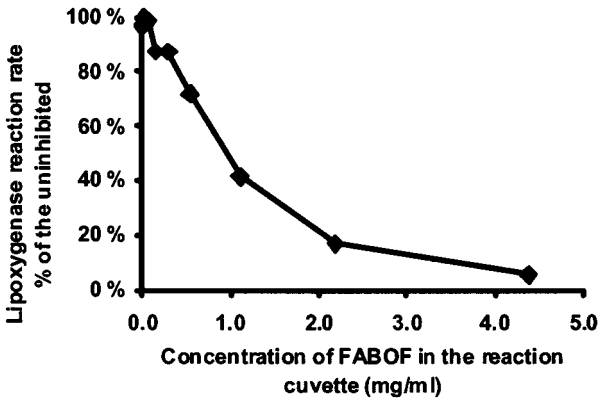

Figure 1. Effect of FABOF on the initial rate of lipoxygenase reaction with 0.12 mg/mL linoleic acid and 0.027 mg/mL enzyme.

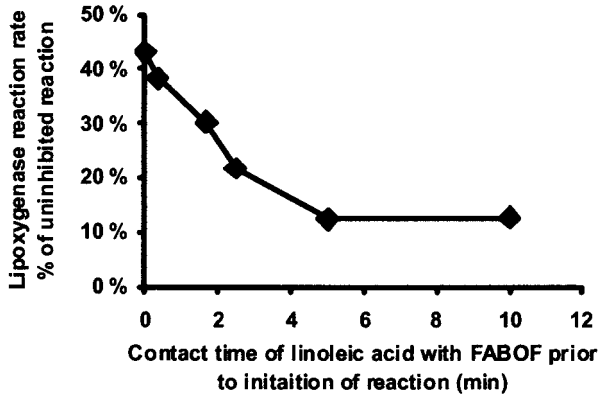

Figure 2. Effect of contact time between the linoleic acid and FABOF on the lipoxygenase reaction rate. Suspension contained 0.12 mg/mL linoleic acid, 0.6 mg/mL FABOF, and 0.027 mg/mL enzyme.

observed lipoxygenase reaction rate would equal half of the rate obtained in the absence of FABOF. This was achieved when FABOF concentration was 0.6 mg/mL, i.e., the amount of FABOF was 5 times the amount of the supplemented linoleic acid.

To elucidate whether the observed reduction in the lipoxygenase reaction rate was due to the interaction of FABOF with the substrate or with the lipoxygenase, FABOF was preincubated with either the substrate or with the lipoxygenase for different periods of time prior to initiating the lipoxygenase reaction. No oxygen consumption was noticed during these preincubation periods, indicating that FABOF does not contain endogenous lipoxygenase activity or linoleic acid capable of acting as a substrate for lipoxygenase. The lipoxygenase reaction rate was not altered upon varying the preincubation time of FABOF with lipoxygenase. However, if FABOF was preincubated with linoleic acid the reaction rate was strongly dependent on the contact time between FABOF and linoleic acid (Figure 2). Even when the reaction was initiated 5 s after mixing linoleic acid and FABOF, the concentration of linoleic acid that serves as a substrate for lipoxygenase had decreased markedly, as judged from the 45% reduction in lipoxygenase reaction rate. The system reached equilibrium after 5 min preincubation by which time the initial lipoxygenase reaction rate was 13% of the uninhibited reaction. This rate of reducing the concentration of oxidizible linoleic acid is much faster than the rate of lipid hydrolysis releasing free fatty acids during cereal processes. In such processes, FABOF would be capable

Table 1. Changes in the Amounts (mg/g FABOF) of Linoleic Acid Present in Different Lipid Classes after Incubation of FABOF with Either Tween-20 Solution or Linoleic Acid/Tween 20 Mixture

	polar lipids	free fatty acids	diglycerides	triglycerides	unextractable
FABOF[a]	11	7	6	29	5
FABOF + Tween[b]	10	5	6	28	5
FABOF + LA/Tween[c]	11	179	6	31	5

[a] Distribution of endogenous linoleic acid in FABOF. [b] FABOF was incubated with Tween-20 solution for 15 min. [c] FABOF was incubated with linoleic acid/Tween-20 solution for 15 min.

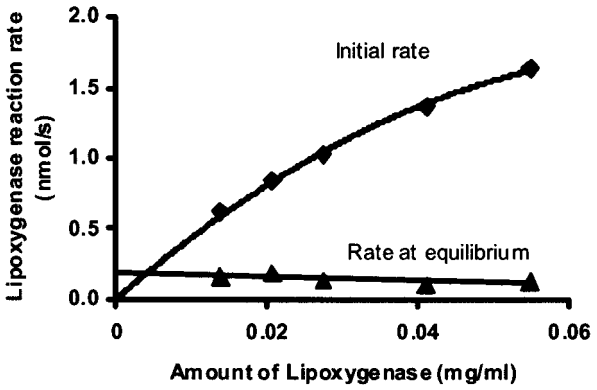

Figure 3. Effect of enzyme concentration on the lipoxygenase reaction rate in the presence of FABOF (0.6 mg/mL) and linoleic acid (0.12 mg/mL). Reaction rate was measured right after the addition of enzyme (◆) and after the steady reaction rate was achieved (▲).

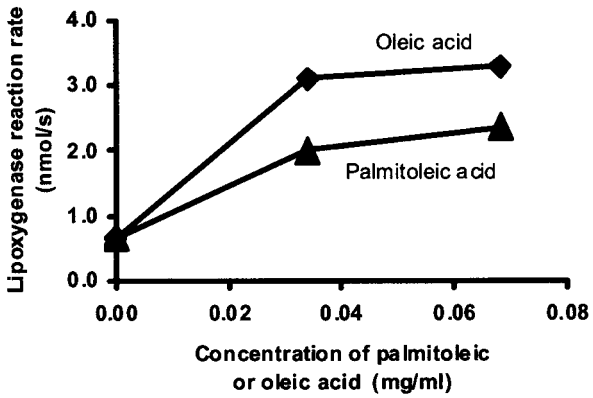

Figure 4. The effect of oleic (◆) or palmitoleic acid (▲) on the rate of linoleic acid oxidation by lipoxygenase. The lipoxygenase reaction contained 0.12 mg/mL linoleic acid, 0.6 mg/mL FABOF, and 0.027 mg/mL LOX.

of interacting with lipid hydrolysis products instantaneously as they are formed, resulting in an effective reduction in the linoleic acid oxidation rate.

When the FABOF/linoleic acid mixture (which had been preincubated for 5 min) was treated with lipoxygenase, a two-phasic reaction velocity curve was obtained. After a rapid initial phase the reaction changed gradually to a slower phase even though the oxygen concentration was still sufficient to maintain the rate of the initial phase. Contradictory to the first reaction phase, the latter phase was unaffected by the changes in the enzyme concentration (Figure 3). This behavior is in accordance with the view that during the preincubation FABOF converts a part of the linoleic acid into the nonmonomer form that does not serve as a substrate for lipoxygenase. The initial reaction rate results from oxidation of the linoleic acid remaining available to lipoxygenase. This pool is consumed quickly, and the latter steady-state phase reflects the rate of dissociation of linoleic acid from the nonreactive to the reactive pool. In the presence of 0.6 mg/mL FABOF and 0.12 mg/mL linoleic acid, the reaction rate in the latter phase, and thus the rate of dissociation of linoleic acid from FABOF, would be 0.14 nmol/s.

In the calculations shown above the formation and dissociation of the nonreactive pool of free fatty acids was assumed to be equal for different free fatty acids. However, it was of interest to monitor whether addition of other fatty acids besides linoleic acid could alter the equilibrium of linoleic acid between the forms unavailable and available to lipoxygenase. For this, FABOF was treated with other free fatty acids dispersed in Tween-20, as described above, prior to the addition of linoleic acid. The addition of a corresponding amount of Tween-20 alone had no effect on the rate of lipoxygenase reaction, i.e., it did not change the amount of linoleic acid FABOF was able to convert unoxidizible. However, if FABOF was treated with a Tween-20 suspension of either oleic or palmitoleic acid prior to supplementing the linoleic acid, the capability of FABOF to reduce the concentration of oxidizible linoleic acid was weakened (Figure 4). This might be due to the competitive binding of the acids to FABOF, because in the absence of FABOF, neither oleic nor palmitoleic acid interfered with the oxidation of linoleic acid by lipoxygenase. Furthermore, the affinity of FABOF was higher toward oleic acid than it was toward palmitoleic acid (Figure 4).

FABOF is a protein- and lipid-rich oat fraction obtained by a procedure that maintains biological activity (Lehtinen and Laakso, 1998). Thus, the possibility that the high transesterification activity of oat is concentrated into FABOF could not be ruled out. If this was the case, then the observed phenomena could be due to esterification of supplemented linoleic acid to endogenous lipids of FABOF, as the lipoxygenase has only limited activity toward acylated linoleic acid. However, analyses of the lipid classes of the preincubated FABOF/linoleic acid mixture revealed that the supplemented linoleic acid was not acylated (Table 1).

CONCLUSIONS

Many cereal processes involve the breakdown of cereal triacylglycerols into free fatty acids. Despite the fact that several cereal extracts are known to inhibit oxidation of polyunsaturated fatty acids (Kähkönen et al., 1999; Lehtinen and Laakso, 1997; Fretzdorff et al., 1986; Nicolas et al., 1981) the hydrolysis of triacylglycerols is considered to predispose fatty acids toward oxidation and further losses in the sensory value of food. The present study demonstrates that the protein-rich fraction of oat reduces the oxidation rate of free linoleic acid by reducing the concentration of linoleic acid that serves as a substrate for lipoxygenase. The mechanism of such behavior appears to be similar to trapping of linoleic acid by cyclodextrin (Lopez-Nicolas et al., 1997). Another possibility is that FABOF stabilizes micelle structures

and thus reduces the concentration of linoleic acid in premicellar forms.

FABOF is a protein-rich oat fraction obtained by an alkaline extraction of oat fiber, and it has been previously published that the total antioxidative capacity originally present in oat fiber is considerably enhanced during this alkaline extraction step (Lehtinen and Laakso, 1998). The alkaline treatment has also been shown to reduce the rate of lipid hydrolysis during fractionating of oat by wet process (Liukkonen et al., 1993). These changes in the functionality upon alkaline treatment may result from the alkaline-induced cross-linking in oat proteins or simply from the increase in the solubility of essential proteins (Finley, 1985; Whitaker, 1980).

LITERATURE CITED

Axelrod, B.; Cheesbrough, T.; Laakso, S. Lipoxygenase from soybeans. In *Methods in Enzymology*; Lowenstein, J. M., Ed.; Academic Press: New York, 1981; Vol. 71.

Chung, O.; Tsen, C. Changes in lipid binding and distribution during dough mixing. *Cereal Chem*. **1975**, *52* (2), pp 533−559.

Delcros, J.; Rakotozafy, L.; Boussard, A.; Davidou, S.; Porte, C.; Potus, J.; Nicolas, J. Effect of mixing conditions on the behavior of lipoxygenase, peroxidase and catalase in wheat flour doughs. *Cereal Chem*. **1998**, *75* (1), 85−93.

Dimberg, L.; Theander, O.; Lingnert, H. Avenanthramides-A group of phenolic antioxidants in oats. *Cereal Chem*. **1993**, *70* (6), 637−641.

Emmons, C.; Peterson, D. Antioxidant activity and phenolic contents of oat groats and hulls. *Cereal Chem*. **1999**, *76* (6), 902−906.

Finley, J. Enviromental effects on protein quality. In *Chemical Changes in Food during Processing*; Richardson, T. and Finley, J., Eds.; AVI Publishing:New York, 1985; pp 443−482.

Frankel, E. Food antioxidants and phytochemicals: present and future perspectives. *Fett/Lipid* **1999**, 101 (12), 450−455.

Fretzdorff, B.; Jördens, A. Vergleichende Untersuchungen zur Substratespezifität, Aktivierung und Inaktivierung von Lipoxygenasen in Getreideextrakten. *Lebensm*. −*Wiss. Technol*. **1986**, *19*, 437−442.

Fritsch, W. Lipid oxidation − the other dimensions. *INFORM (Champaign, Ill)* **1994**, 5 (4), 423−436.

Galpin, J.; Allen, J. The influence of micelle formation on lipoxygenase kinetics. *Biochim. Biophys.Acta* **1977**, *488*, 392−401.

Graveland, A. Enzymatic oxidation of linoleic acid and glyserol-1-monolinoleate in doughs and flour-water suspensions. *J. Am. Oil Chem. Soc*. **1970**, 47 (9), 352−361.

Kähkönen, M.; Hopia, A.; Vuorela, H.; Rauha, J.-P.; Pihlaja, K.; Kujala, T.; Heinonen, M. Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. *J. Agric. Food Chem*. **1999**, *47* (10), 3954−3962.

Lagocki, J.; Emken, E.; Law, J.; Kézdy, J. Kinetic analysis of the action of soybean lipoxygenase on linoleic acid. *J. Biol. Chem*. **1976**, *19*, 6001−6006.

Lehtinen, P.; Laakso, S. Antioxidative-like effect of different cereals and cereal fractions in aqueous suspension. *J. Agric. Food Chem*. **1997**, *45*, 4606−4611.

Lehtinen, P.; Laakso, S. Effect of extraction conditions on the recovery and potency of antioxidants in oat fiber. *J. Agric. Food Chem*. **1998**, *46*, 4842−4845.

Liukkonen, K. H.; Montfoort, A.; Laakso, S. Water-induced lipid changes in oat processing. *J. Agric. Food Chem*. **1992**, *40* (1), 126−130.

Liukkonen, K.; Kaukovirta, N.; Laakso, S. Elimination of lipid hydrolysis in aqueous suspensions of oat flour. *J. Cereal Sci*. **1993**, *17*, 255−265.

Lopez-Nicolas, J. M.; Bru, R.; Garcia-Carmona, F. Enzymic Oxidation of Linoleic Acid by Lipoxygenase Forming Inclusion Complexes with Cyclodextrins as Starch Model Molecules. *J. Agric. Food Chem*. **1997**, *45*, 1144−1148.

Ma, C.; Harwalkar, V. Chemical characterization and functionality assessment of oat protein fractions. *J. Agric. Food Chem*. **1984**, *32*, 144−149.

Mann, D. L.; Morrison, W. R. Effects of ingredients on the oxidation of linoleic acid by lipoxygenase in bread doughs. *J. Sci. Food Agric*. **1975**, *26*, 493−505.

Nicholas, J.; Autran, M.; Drapron, R. Quelques carctéristiques de l'inhibiteur de l'activite lipoxygénàsique presént dans la farine de blé tendre. *Sci. Aliments* **1981**, *1* (2), 213−232.

Suutari, M.; Liukkonen, K.; Laakso, S. Temperature adaptation in yeast: The role of fatty acids. *J. Gen. Microbiol*. **1990**, *136*, 1469−1474.

Tian, L. L.; White, P. J. Antioxidant activity of oat extracts in soybean and cottonseed oils. *J. Am. Oil Chem. Soc*. **1994**, *71* (10), 1079−1086.

Whitaker, J. Changes occurring in proteins in alkaline solution. In: *Chemical Deterioration of Proteins*, J. R. Whitaker and M. Fujimaki Eds.; ACS Symposium Series 123; American Chemical Society: Washington, DC, **1980**; pp 145−163.

Youngs, V. Oat lipids. *Cereal Chem*. **1978**, *55*, 591−597.

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