Oxidation of Various Lipid Substrates with Unfractionated Soybean and Wheat Lipoxidase

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Abstract

Quantitative determinations of lipoxidase in wheat were made on five milling fractions from four wheat varieties, including two hard red winter and two hard red spring wheats. Lipoxidase content decreased with increasing refinement and was lowest in the flour fraction. In general, the mill fractions from spring wheats exhibited slightly higher lipoxidase content than the corresponding mill fractions from winter wheats.

Aqueous extracts from soybeans (variety Hawkeye) and wheat (variety Selkirk, break shorts fraction) were adjusted in concentration so that the lipoxidase activity with respect to linoleic acid was approximately the same. The diluted extracts were then tested for their ability to oxidize other lipid substrates with the necessary 1,4 pentadiene system. Soybean lipoxidase was far more reactive toward methyl linoleate and trilinolein than wheat lipoxidase. Mono- and dilinolein were relatively unreactive in both systems although dilinolein was a better substrate in the soybean system than in the wheat. Neither system oxidized highly unsaturated digalactosyl diglycerides alone. However the soybean extract oxidized this substrate to a small extent in the presence of catalytic amounts of linoleic acid while, under identical conditions, the wheat extract remained inactive.

Polyacrylamide gel electrophoresis of the aqueous extracts of soybeans and wheat-milling fractions coupled with a staining procedure specifically for lipoxidase indicated three to four isoenzyme bands in the soybeans and two to four isoenzyme bands in the wheat-milling fractions. Isoenzymes may be responsible for differences in enzymatic activity on various substrates.

Introduction

In studies on the uptake of oxygen by flour dough, Smith and Andrews (1) showed that much of the oxygen incorporated into doughs is the result of the lipoxidase-polyunsaturated lipid system. These workers concluded that the free lipid and, more specifically, the free fatty acids were the main substrate involved. Tsen and Hlynka (2) confirmed these observations and proved that fatty acid hydroperoxides were produced during the mixing of doughs. They further showed that the addition of several peroxides and hydroperoxides, including oxidized linoleic acid, strengthened doughs as evidenced by an increased relaxation constant.

The effects of added lipoxidase (in the form of an enzyme-active soybean flour preparation) on the rheological properties of doughs have been investigated. Koch (3) found that, in the large majority of flours tested, the addition of 1% commercial lipoxidase preparation increased the mixing tolerance of the dough according to farinographic analysis. Smith and Andrews (1) were unable to confirm this effect and, in fact, observed a decrease in tolerance with the addition of a similar lipoxidase preparation. However these authors do suggest that it is possible that lipoxidase may act to increase mixing tolerance of doughs under certain conditions. Prior to these reports Logan and Learmonth (4) reported that added soybean flour increased gluten oxidation in doughs. As a result of these and other observations concerning the bleaching capabilities of soybean flour, several processes for improving bread, involving the addition of a small amount of soybean flour to the wheat flour, have been patented or proposed (5–7).

Although it cannot be denied that the lipoxidase system is the primary source of incorporated oxygen in doughs, it has been shown that only a small amount of oxygen is needed for dough improvement. Thus Hawthorn (8) has calculated that the level of oxygen uptake in flour-water mixtures, observed by Smith and Andrews (1), is around 100 times the level required of typical chemical improvers in terms of oxygen equivalents. Furthermore defatted flours, mixed by a high speed process, resulted in breads of increased volume over defatted controls mixed in a more conventional manner (9). The results of these and other experiments have led to the theory that, as an improver, oxygen acts in a more direct manner- possibly by direct oxidation of –SH or other easily oxidized centers in the dough (8, 10).

Since the oxygen absorption capabilities of doughs with the use of endogenous lipoxidase would seem to be more than sufficient (in terms of oxygen equivalents) for improvement, the question arises why an additional source of lipoxidase should so affect dough improvement. One reason may be that soybean lipoxidase attacks more readily than wheat lipoxidase substrates other than the free fatty acids, e.g., triglycerides. It is the purpose of this paper to describe some comparative studies of the reactivity of wheat and soybean lipoxidase with a variety of possible substrates which possess the necessary 1,4-pentadiene system.

Experimental Procedures

All chemicals used were reagent grade, and water was redistilled from glass. Linoleic acid, methyl linoleate, mono-, di-, and trilinolein were obtained from the Hormel Institute, Austin, Minn., and were greater than 99% pure. Digalactosyl diglycerides were a gift of D. V. Myhre of the Procter and Gamble Company, Cincinnati, Ohio.

Transesterification of the digalactosyl diglycerides was performed by the method of Morrison and Smith (11), and gas-liquid chromatography (GLC) of the methyl esters was carried out on an Aerograph Model 1520 gas chromatograph, equipped with a hydrogen flame detector. The esters were separated on a 5-ft by ⅛-in. stainless steel column, containing 15% diethyleneglycol succinate on acid-washed Chromosorb

W. Peaks were quantified by multiplying peak height by the width at half-height.

Protein was determined on the TCA precipitates of the crude extracts by using the microbiuret method of Goa as described by Bailey (12) with bovine serum albumin as the standard.

Quantitative estimation of lipoxidase activity in the various mill fractions was performed by the method of Surrey (13) on fresh aqueous extracts, prepared by mixing 1 g of sample with 25 ml of water at 4C for 1 hr with gentle agitation, provided by a magnetic stirrer.

For comparative studies on various substrates, crude extracts of wheat and soybeans were made as described earlier (14). The source of wheat lipoxidase was a break shorts fraction from variety Selkirk. This was the richest source available, and earlier studies indicated no significant qualitative differences between the lipoxidase in this fraction and that of the other wheat fractions. A purified soybean lipoxidase preparation was obtained from the Sigma Chemical Company, St. Louis, Mo.

For the comparative studies, lipoxidase activity was determined manometrically under oxygen by means of a Gilson recording differential respirometer. Reactions were carried out at 30C in 0.067 M phosphate buffer at pH 6.6. Reaction volumes were 1.5 ml, and substrate concentration was 5×10^{-3} M, based upon the amount of 1,4 pentadiene present unless otherwise stated. Reaction rates were determined from the initial zero-order portion of the rate curves. Substrates were prepared as described previously (14).

Results

Lipoxidase Concentration

In Table I the results of the quantitative studies of lipoxidase activity in the various mill fractions of four wheat varieties are compiled. Since these data are based on the concentration of conjugated diene hydroperoxide, the values must be considered as minimal in view of the recent report by Zimmerman (15) concerning the presence of a hydroperoxide isomerase in flaxseed. The presence or absence of this enzyme was not investigated in these wheat mill fractions.

In qualitative agreement with Miller and Kummerow (16) there is decreasing activity with increasing refinement. The over-all data indicate that there may be a slightly higher concentration of lipoxidase in mill fractions from spring wheat (Lee and Selkirk) than in winter (Bison and Triumph). Similarly Honold and Stahmann (17) found higher concentrations of catalase and peroxidase in spring wheat than in winter wheat in the same mill fractions studied in this paper.

The relatively high value for Lee flour is probably an anomaly since this flour also had poor baking qualities and anomalous proximate analysis when compared with a freshly milled flour from Lee whole wheat (18).

TABLE I

Lipoxidase Activity of Various Mill Fractions from Four Varieties of Wheat

Fraction	Lipoxidase Activity[a] (units/g)			
	Bison	Triumph	Selkirk	Lee
Break shorts	71.2	59.2	110	91.7
Reduction shorts	50.0	36.5	54.7	61.0
Red Dog	42.2	30.2	35.2	60.2
Bran	20.7	22.0	42.5	27.4
Flour	3.2	2.0	6.2	21.7

[a] One unit of activity was defined as that activity which produced an optical density of 0.1, at 234 mμ in 1 minute, in a total volume of 10 ml of 60% ethanol solution (13).

TABLE II

Effect of Frozen Storage on Lipoxidase Activity in Crude Soybean and Wheat Extracts[a]

Source	Age of extract (months)	Oxygen absorption (μl/min)
Wheat	0	10.7 ± 0.1
	4.5	9.5 ± 0.2
Soy	0	9.1 ± 0.2
	4.5	9.8 ± 0.5

[a] Extracts stored at −20C.

Storage Stability

Single extraction of the Selkirk break shorts fraction and the soybeans were used throughout this work. The data in Table II show the stability of the lipoxidase in these preparations when stored at −20C. A slight decrease in the activity of the wheat enzyme is evident whereas the activity of the soybean lipoxidase is not changed after 4.5 months. Many of the data reported in this paper were collected within three months after the extractions were made.

The universal substrate used in measuring lipoxidase activity is linoleic acid or its salt. For the purpose of comparing with other substrates, appropriate dilutions for the crude wheat and soybean extracts were done to determine that their activity toward linoleic acid was approximately the same. The data in Table II show the activity of the 0.2 ml aliquots of the diluted extract which was used throughout this study. The protein concentration of the diluted wheat extract was 1.76 mg/ml, and that of the diluted soybean extract was 0.56 mg/ml.

Trilinolein and Methyl Linoleate

It was originally intended that the various substrates in this investigation would be made up in 1% albumin in order to obtain more stable emulsions. When linoleic acid systems with and without added albumin were compared, essentially no differences in oxidation rates were obtained with either the soybean or wheat extracts. However Fig. 1 shows that the oxygen uptake with trilinolein as the substrate was greatly reduced in the presence of 1% albumin. It

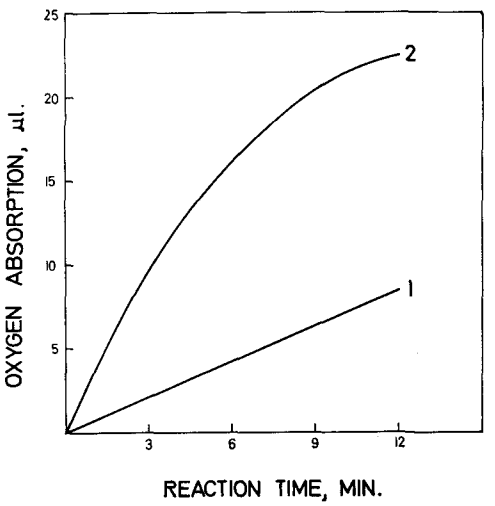

FIG. 1. Effect of albumin on the catalytic oxidation of trilinolein by crude soybean extract: 1. trilinolein + 1% albumin, 2. trilinolein only.

may be that the albumin forms a complex with the trilinolein, making it unavailable for oxidation by lipoxidase. No further data were collected concerning this phenomenon, but, in view of these results, the use of albumin in reaction mixtures was abandoned.

The data in Table III show that methyl linoleate and trilinolein were rapidly oxidized in the crude soybean system; in fact, under these conditions, the methyl linoleate was more rapidly oxidized than the free fatty acid. The crude wheat system could oxidize methyl linoleate to some extent, but this system was almost unreactive toward the trilinolein substrate. These data suggest a fundamental difference in the lipoxidase systems of soybeans and wheat.

It has been demonstrated that soybeans contain at least two lipoxidase enzymes, i.e., a fatty acid lipoxidase and a triglyceride lipoxidase (19,20). Koch et al. (19) found that, when these two enzymes were separated, the fatty acid lipoxidase had only slight activity with methyl linoleate as the substrate. With the triglyceride enzyme the rate of oxidation of trilinolein was about two times greater than that of methyl linoleate. Under the conditions of this investigation where both the fatty acid and triglyceride lipoxidase are present, methyl linoleate is more rapidly oxidized than either linoleic acid or trilinolein. There may be a synergistic effect between the fatty acid and triglyceride lipoxidases in the case where methyl linoleate is the substrate.

Commercially purified soybean lipoxidase was adjusted in concentration so that its activity with the linoleic acid substrate approximated that of the crude soy and wheat extracts. Table III shows the activity of this preparation with the linoleic acid, methyl linoleate, and trilinolein substrates. It is clear that the purified enzyme is of the fatty acid variety, and it is interesting to note the similarities in the oxidation rates of the purified soy enzyme and the crude wheat extract.

By using polyacrylamide gel electrophoresis and a procedure described earlier (14), the lipoxidase isoenzyme patterns of the various enzyme sources in this investigation were compared. The patterns obtained with wheat, unfractionated soybean, Wytase (a commercial flour-improver derived primarily from soybean flour), and purified soybean lipoxidase are shown in Fig. 2. The primary activity bands from all sources have similar electrophoretic properties; the broad band from the soybean sources encompasses the same R_f range as the two main bands from wheat. The primary difference to be noted in these patterns is that the crude soybean (and Wytase) extract exhibits a prominent activity band at the origin or cathode end of the small pore gel whereas the purified soybean and crude wheat lipoxidase no not show activity in this region. Whether or not this slow migrating band in the crude soybean extract represents the triglyceride lipoxidase has not been

TABLE III

Oxygen Absorption by Wheat and Soybean Extracts and Purified Soybean Lipoxidase with Various Lipid Substrates

	Oxygen Absorption (μl/min)		
Substrate	Soybean extract	Wheat extract	Purified soybean lipoxidase
Linoleic acid	9.1 ± 0.2	10.7 ± 0.1	9.5 ± 0.6
Methyl linoleate	11.2 ± 0.7	1.2 ± 0.1	1.8 ± 0.2
Trilinolein	3.8 ± 0.3	0.3 ± 0.1	0.3 ± 0.0
Dilinolein	0.8 ± 0.0	0.3 ± 0.0	.....
Monolinolein	0.1 ± 0.0	0.4 ± 0.1	.....

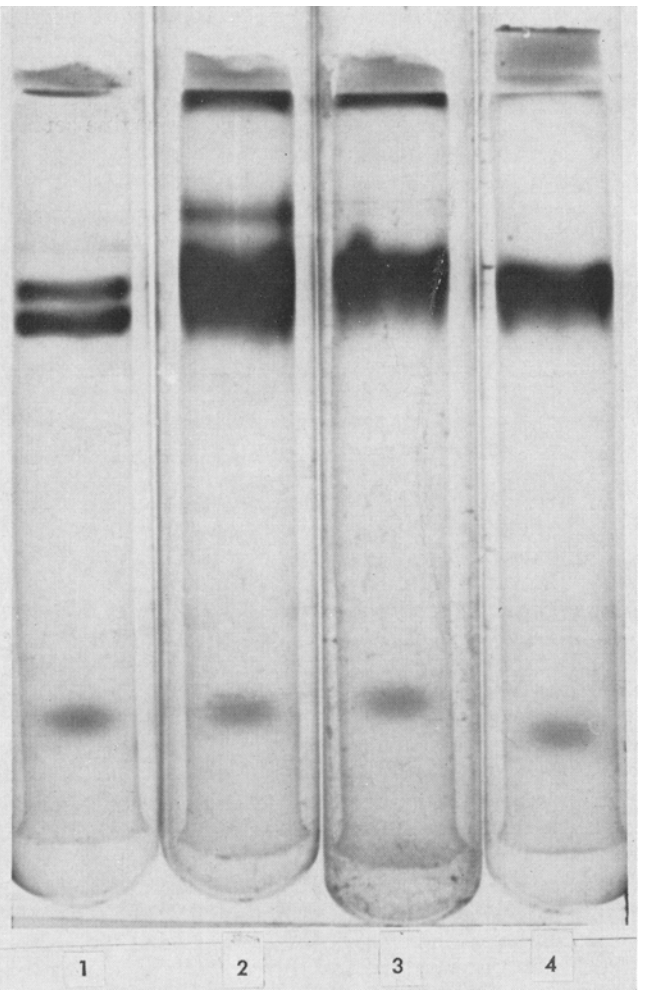

FIG. 2. Polyacrylamide gel zymograms of lipoxidase isoenzymes: 1. wheat extract, 2. soybean extract, 3. Wytase extract, 4. purified soybean lipoxidase.

determined. Attempts to demonstrate triglyceride lipoxidase activity on polyacrylamide gels with trilinolein substrate have thus far been unsuccessful, probably owing to the inability of the substrate to diffuse into the gel.

Mono- and Dilinolein

The data in Table III show that neither mono- nor dilinolein was a good substrate in either the wheat or soybean system. The wheat extract exhibited constant activity toward all glyceride substrates, and the soybean system showed decreasing activity with increasing polarity of the glyceride substrates.

The monolinolein produced the most finely dispersed and stable substrate of the glycerides but was the least reactive in the soybean system. This low activity may be the result of a sensitivity of the triglyceride lipoxidase toward surfactants. Dillard et al. (20) found that the triglyceride lipoxidase from soybeans was extremely sensitive to Tween 40 for it was completely inactivated at a surfactant concentration of about 2.5 mg/100 ml. They also indicated that the activity of the fatty acid lipoxidase was actually enhanced up to a Tween 40 concentration of 7.5 mg/100 ml, after which inhibition was observed with higher surfactant concentration. Similarly the higher but still low activity with dilinolein may also be caused by the slight surfactant effects of this substrate. Alternatively the orientation of the hydroxyl groups at the

substrate interface may hinder substrate-enzyme interaction in the case of mono- and dilinolein.

Digalactosyl Diglycerides

The primary component of wheat flour polar lipids is the digalactosyl diglycerides (DGDG). These particular lipids account for approximately 40% of the polar lipids in flour (21,22). Since the DGDG are known to contain high levels of polyunsaturated fatty acids (21), it was of interest to observe their effectiveness as lipoxidase substrates with the soybean and wheat extracts. It should be noted that this DGDG preparation contained a small but unknown amount of cerebroside. Prior to their use as substrates, GLC was performed on the methyl esters of the DGDG fatty acids to determine the amount of polyunsaturation present, hence the amount of possible substrate. Esterification yields indicated that the DGDG contained 52.8% fatty acids, and Table IV shows that linoleic acid was the primary fatty acid, comprising 68.6% of the total fatty acids. The only other possible lipoxidase substrate present was linolenic acid, which made up 3.9% of the total fatty acids.

The DGDG substrate was so made up that the concentration of pentadiene was equivalent to 5×10^{-3} M linoleic acid. Initial experiments, employing only DGDG plus enzyme, showed virtually no catalytic oxidation of the substrate by either the soybean or wheat extracts.

A subsequent series of experiments was set up with the DGDG substrate at the same concentration but with the addition of catalytic quantities (10^{-4} M) of linoleic acid. Fig. 3 gives the results of one of these experiments with the use of the crude soybean extract. Reaction Curve 4 represents the enzymatic oxidation of 1.3×10^{-3} M linoleic acid only and is placed in this figure for comparison; it confirms that the normal lipoxidase system is functioning properly. The data show a definite enhancement of the oxygen uptake by the added linoleic acid, and calculations indicate that, after 30 minutes, more oxygen is absorbed than would be accounted for if only the linoleic acid were being oxidized. At a linoleic acid concentration of 5×10^{-5} M, no enhancement of oxygen uptake was observed with the DGDG substrate.

The wheat extract did not respond in a similar manner with the normal DGDG substrate plus 10^{-4} M linoleic acid. These data again suggest differences in the lipid-oxidizing capabilities of soybean and wheat enzymes.

Whether or not linoleic acid is acting as a coenzyme in Reaction 3 of Fig. 3 cannot be established from the data presented here, and the limited amount of DGDG available did not allow further elucidation of the mechanism. It is not likely that the observed increase in oxygen absorption is owing to autoxidation of the DGDG which is catalyzed by the linoleic acid hydroperoxides produced by lipoxidase. If this were the case, the wheat system should have given similar results.

TABLE IV
Fatty Acid Composition of Digalactosyl Diglycerides

Fatty acid[a]	Weight percentage
16:0	15.2
16:1	0.9
18:0	2.2
18:1	9.2
18:2	68.6
18:3	3.9

[a] Carbon number: double bonds.

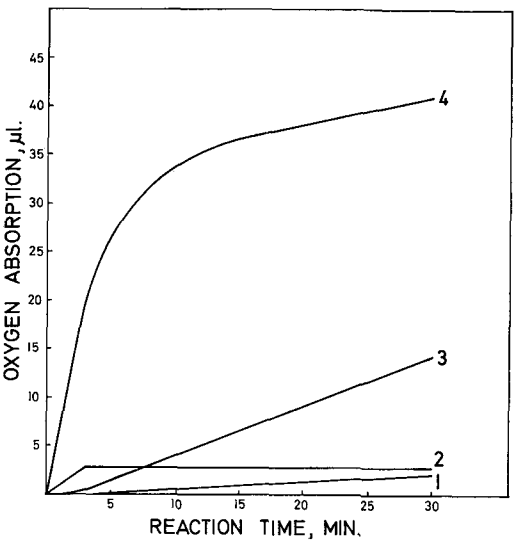

FIG. 3. Effect of catalytic quantities of linoleic acid on oxidation of digalactosyl diglyceride (DGDG) oxidation by soybean extract: 1. DGDG only, 2. 10^{-4} M linoleic acid only, 3. DGDG + 10^{-4} M linoleic acid, 4. 1.3×10^{-3} M linoleic acid only.

In an experiment confirming the results in Fig. 3, two reaction mixtures containing 10^{-4} M oleic acid in place of linoleic acid were run. There was no enhanced oxidation of the DGDG by added oleic acid, which would argue against the physical effects of linoleic acid as the cause for the enhanced oxidation.

Discussion

It has been known for some time that there are apparently two different lipoxidase enzymes, the fatty acid lipoxidase and the triglyceride lipoxidase (19, 20). Smith and Andrews (1) have shown that the rate of oxygen uptake is linearly related to the free fatty acid content in flour-water mixtures. They also found that flour triglycerides supported some oxygen consumption in doughs, but they suggested that this was small compared with the free fatty acids. Their data and the results in this paper would indicate that the triglyceride lipoxidase is absent in wheat.

The multiplicity of lipoxidase isoenzymes in the wheat extract is interesting in view of the fact that this system apparently has only fatty acid lipoxidase activity. A possible explanation for the existence of fatty acid lipoxidase isoenzymes might be noted in the recent work of Dolev et al. (23). They found one crystalline lipoxidase preparation to be highly specific, forming only the 13-hydroperoxyoctadecadienoic derivative from linoleic acid. However a second crystalline preparation from the same supplier yielded both the 9- and 13-hydroperoxyoctadecadienoic fatty acid derivatives. Similarly Hamberg and Samuelsson (24) obtained a mixture of 70% 13- and 30% 9-hydroperoxyoctadecadienoic fatty acid from the reaction of linoleic acid and purified lipoxidase. These results would suggest that there may be at least two fatty acid lipoxidases with positional specificity; and it would not be unlikely that these isoenzymes would behave differently on polyacrylamide gel electrophoresis.

The question whether or not added soybean lipoxidase preparations significantly aid in the oxidative improvement of doughs has not been fully answered. The data of Tsen and Hlynka (2) show that the addition of several hydroperoxides and peroxides has

a beneficial effect on certain dough rheological properties, indicating a similar role for lipid hydroperoxides. Hawthorn (8) cites evidence however from his laboratory, indicating that known lipoxidase inhibitors (NDGA and α-tocopherol acetate), when added to doughs, do inhibit the bleaching process but do not produce a concurrent loss of loaf volume in the high-speed stage of the Rank and Hay process (5). Thus the improving mechanism in this process may not be caused by the action of lipoxidase. The data in this paper are not intended to shed light one way or the other on this question but rather are intended to point out that, with the addition of unfractionated soybean lipoxidase preparations, the scope of possible enzymatically catalyzed lipid peroxidation in doughs is broadened to include the triglycerides and possibly the polar lipids.

The role of polar lipids in bread quality may be of the greatest importance. Thus Pomeranz et al. (25) have shown that, at a level of 0.5% of the flour, unfractionated flour polar lipids give an improvement in loaf volume and crumb grain approximately equal to that gained by the addition of 3% vegetable shortening. Recently Daniels et al. (26) have presented data to indicate that polar lipids with a high degree of polyunsaturation are perferentially bound over more saturated polar lipids during mixing and especially during the baking process. Neither of the above authors suggest that their results are attributable to an oxidative process.

Investigations into the relative contributions of the fatty acid and triglyceride lipoxidases in those processes which use soy flour preparations as a dough improver might be useful in elucidating the lipoxidase-polyunsaturated lipid-protein system in doughs.

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