Lipoxygenase: Its Biochemistry and Role in Breadmaking[1]

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ABSTRACT

The biochemistry of the enzyme lipoxygenase and its effect on wheat flour dough are reviewed. Lipoxygenase has a number of effects on wheat flour dough. It is quite effective as a bleaching agent, increases the mixing tolerance, and improves dough rheology. The bleaching action is thought to be the coupled oxidation of pigments and unsaturated fatty acids by atmospheric oxygen. The mechanism by which lipoxygenase increases mixing tolerance is not clear. Because lipoxygenase has no effect on defatted flour, lipids are clearly involved. Lipid extracted from lipoxygenase-treated dough has been reported to decrease mixing tolerance when added back to flour. The improving effect of lipoxygenase on dough rheology has been explained as an oxidation of sulfhydryl groups by the lipid peroxides formed by the action of lipoxygenase on lipid. However, recently reported work has shown that the inhibitor nordihydroguaretic acid greatly inhibited peroxide formation but only marginally impaired rheological effects. Thus, the effect of peroxides is questionable.

The enzyme lipoxygenase (E.C. 1.13.11.12) is a dioxygenase that catalyzes the oxidation of polyunsaturated fatty acids to hydroperoxides. Several reports have reviewed the physical and biochemical aspects of the enzyme's mechanism (Axelrod 1974, Morrison 1978a, Veldink et al 1977) and its numerous effects on food systems (Eskin et al 1977). This review discusses our current understanding of the biochemistry of lipoxygenase and its role in breadmaking.

BIOCHEMISTRY OF LIPOXYGENASE

Sources of Lipoxygenase

Soybeans are the richest and best known source of lipoxygenase, which is widely distributed in the plant kingdom; Axelrod (1974) lists 38 sources. In addition, the enzyme recently has been demonstrated in animal tissue (Nugteren 1975, Samuelsson 1972). Other sources studied include corn (Veldink et al 1972), alfalfa (Chang et al 1972), peas (Yoon and Klein 1979) and wheat (Wallace and Wheeler 1975). Researchers recognized early that soybean flour not only bleached wheat flour pigments (Haas and Bohn 1934), but also oxidized the fat extracted from unboiled soybean curd (Andre and Hou 1932) and that both phenomena required oxygen. Work by two groups (Sumner and Sumner 1940, Tauber 1940) showed that lipid oxidase and bleaching activity stemmed from the same enzyme. Peroxidation of unsaturated lipid was the primary reaction, whereas pigment bleaching was apparently a coupled reaction occurring only during enzymatic attack on lipid. Theorell's isolation and crystallization of lipoxygenase (now known to be the isoenzyme-1) from soybean (Theorell et al 1947), conclusively demonstrated the proteinaceous and enzymatic character of lipoxygenase.

Isolation and Purification

The methods used to purify lipoxygenase are important. In most sources examined, the enzyme exists in multiple forms or isoenzymes: four in soy (Axelrod 1974), wheat (Wallace and Wheeler 1975), and peas (Yoon and Klein 1979) and two in corn (Veldink et al 1972), and they differ in several important properties including pH optimum, bleaching capacity, substrate specificity, and products produced. Thus, failure to completely separate the isoenzymes, or misidentifying one can lead to serious mistakes in interpreting results.

Regardless of source, the isolated enzymes are polypeptides of between 95,000 and 100,000 mol wt. The enzyme was thought to be unique among oxygenases in that it lacked prosthetic groups. However, Chan (1973) reported 1 mole of iron per mole of enzyme for soybean lipoxygenase, which is a common property of all lipoxygenases.

Reaction Mechanism

In discussing the reaction mechanism and products of lipoxygenase, one has difficulty not becoming caught up in a litany of differing products from differing isoenzymes under differing experimental conditions. The problem is compounded by the fact that lipoxygenase reacts under anaerobic as well as aerobic conditions. Recent work (DeGroot et al 1975, Vliegenthart et al 1979) produced tenative models of the lipoxygenase mechanisms; an appreciation of those reactions is important to understanding enzymatic lipid oxidation in food systems, so a brief overview is justified.

Substrate Specificity

As shown in Fig. 1, a common feature of all lipoxygenases is their attack on fatty acids possessing *cis,cis*-1,4-pentadiene unsaturation (Hamburg and Samuelsson 1967, Holman et al 1969). Beyond that strict requirement and a preference for linoleic acid, lipoxygenase substrate specificity is difficult to discuss. Wide differences in the solubilities of the enzyme and substrate make the actual amount of substrate available to the enzyme uncertain. The problem is compounded when linoleic acid is esterified in such diverse entities as phospholipids and mono-, di-, or triglycerides.

As might be expected, results differ according to the enzyme source and assay system used. Purification of lipoxygenase-1 from soy (Holman et al 1969) requires that the substrate fatty acid be sterically unhindered at the carboxyl end. A soy lipoxygenase has been proposed (Koch et al 1958) that attacks triglyceride and has a pH optima different from that of lipoxygenase-1. Crude soy lipoxygenase is 10 times more active with methyl linoleate and trilinolein than is crude wheat lipoxygenase, whereas both preparations have similar activity with linoleic acid (Guss et al 1968).

In an article reporting extraction and analysis of lipids from small quantities of flour and dough, Graveland (1968) found that dough could be used to study lipoxygenase. He used the technique (Graveland 1968, 1970) to detect wheat lipoxygenase activity with linoleic and linolenic acids as well as with glycerol monolinoleate.

Recent work (Mann and Morrison 1974, 1975) has confirmed the ability of wheat lipoxygenase to oxidize free linoleic or linolenic acids and their monoglycerides. When tested in dough systems (Morrison and Panpaprai 1975), soy lipoxygenase was active with linoleic or linolenic acid in mono- and digalactosyldiglycerides.

Specificity Of Product Produced

After enzyme and substrate have bound, the reaction is initiated

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by the abstraction of a hydrogen from the C-8 methylene group of the linoleic acid substrate. The stereospecificity of this reaction apparently depends on enzyme source. It takes place in both the presence and absence of oxygen and occurs via a free radical mechanism, as shown by electron paramagnetic resonance spectroscopy and spin traps (DeGroot et al 1973, Walker 1963). The radicals formed are apparently on the fatty acid molecule. The reactivity of this radical and its subsequent peroxy radical are important in lipoxygenase activity.

Once the radical is formed, it is free to stabilize by resonance delocalization over the pentadiene system (Fig. 1). The second stage of the aerobic reaction is oxygenation by molecular oxygen (Dolev et al 1966) of the fatty acid radical. The resulting compound is the optically active 9D or 13L hydroperoxide of linoleic acid (Privett et al 1955), referred to as 9 or 13 LAHPO.

The ratio of 9 to 13 LAHPO enzymatically produced by a given lipoxygenase is termed its positional specificity, which varies among enzyme sources; it is also known to be affected by the substrate as well as the temperature and oxygen tension of the assay medium (Eskin et al 1977, Privett et al 1955). Finally, differences in positional specificities exist between lipoxygenase isoenzymes exposed to the same substrate.[4] A concise tabular summary of these effects and the resulting products of the enzyme are given by Gaillard (1975) and Axelrod (1974).

The remaining reaction path for lipoxygenase (Fig. 2) has been termed the anaerobic reaction, which has been studied exclusively with soybean isoenzyme-l. Anaerobic is somewhat of a misnomer because no reaction occurs when the native enzyme is incubated anaerobically with linoleic acid (Vliegenthart and Veldink 1977). The anaerobic reaction refers to changes that occur in substrate linoleic acid when it is incubated with lioxygenase under either of two conditions (Garssen et al 1971): with O_2 in limiting concentrations or anaerobically with preformed LAHPO. The resulting products are dimers of the substrate and oxodienoic acids (Garssen et al 1972). Apparently a limiting amount of O_2 does nothing more than create the requisite hydroperoxides. The mechanism clearly involves fatty acid radicals in solution due to dissociation of the enzyme-radical complex (Veldink et al 1977). The proposed mechanism (Fig. 3) contends that fatty acid dimers arise by 1) a termination reaction between two fatty acid radicals or 2) a chain reaction of radical with free lipid. Alkoxy radicals would similarly lead to oxygen containing dimers.

[4] J. Christopher. 1972. Unpublished results cited in Axelrod (1974).

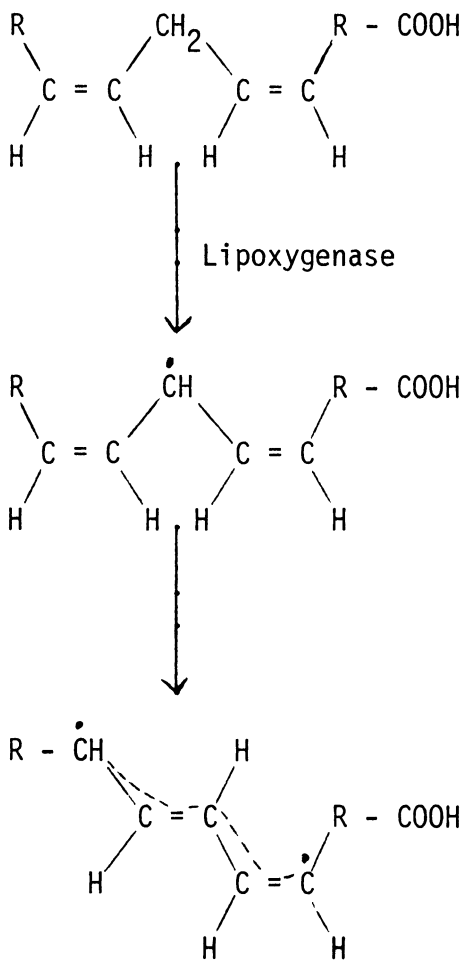

Fig. 1. Top, *cis, cis*-1,4 unsaturation necessary for lipoxygenase activity. **Bottom,** resonance delocalization of the resulting lipid radical. Dots at the 9 and 13 carbons show the two possible sites for O_2 attack.

Thus, the primary products of lipoxygenase action are fatty acid hydroperoxides or fatty acid dimers. Secondary products include the breakdown of hydroperoxide decomposition products—of interest to food scientists but outside the scope of this review. Bleached pigments also can be considered secondary products of lipoxygenase action.

DeGroot et al (1975) published a model for soy lipoxygenase-l activity that Vliegenthart and Veldink expanded in 1977. The model fits well with existing knowledge of the enzyme.

In the DeGroot model, the interaction of one molecule of hydroperoxide with the inactive native enzyme (step 1) yields an active enzyme, presumably via a single electron transfer from iron to oxygen, which would be released in the form of activated oxygen, or to hydroperoxide, which would be released as RO• and OH^-.

In the aerobic reaction, hydroperoxide is converted to as yet unidentified compounds, perhaps by reacting with activated oxygen. The Fe^{+3}-Enz is reduced by the bound fatty acid substrate with formation of the linoleate free radical (step 2). Oxygen stereospecifically combines with the free radical-enzyme (step 3), followed by a single electron transfer from iron to the peroxy radical, regenerating the active enzyme and releasing the peroxy anion, which is then protonated.

Anaerobically, the enzyme-radical complex is activated by product hydroperoxide. Reduction by substrate is identical with the aerobic process, but in the absence of molecular oxygen, the enzyme-radical complex dissociates, giving free E-Fe^{+++} and R•. The active enzyme is regenerated by interaction with product hydroperoxide to give alkoxy free radicals RO• and OH^-. The reactive radical species combine as outlined earlier.

Co-Oxidation

The ability of lipoxygenase to bleach or decolorize several pigments is one of its oldest, but ironically its least understood, characteristics. Work on the enzyme from a variety of sources has shown that substrates for bleaching vary and include carotene (Sumner and Sumner 1940), xanthophylls (Bohn and Haas 1953),

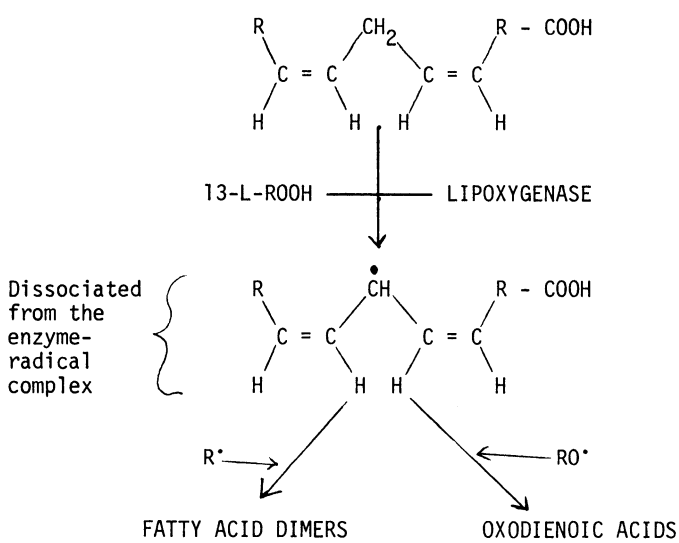

Fig. 2. Anaerobic reaction of soy lipoxygenase isoenzyme-l.

bixin (Sumner and Smith 1947), chlorophyll (Yoon and Klein 1979), cholesterol (Teng and Smith 1972), crocin (Grosh et al 1977, Weber et al 1974), lutein (McDonald 1979), and various dyes (Drapron and Nicolas 1977, Ikediobi 1977).

Despite all that work, no coherent mechanism or theory exists to explain bleaching, probably because lipid oxidation and bleaching, although linked, are not the same activities. The initial studies identifying isoenzymes in the soybean system were prompted by observing that bleaching and hydroperoxide formation showed different stabilities to heat treatment in crude extract (Kies 1947). Kies et al (1969) later recognized that cooxidizing abilities varied among isoenzymes and that the variation did not parallel differences in lipid-oxidizing activity. The most widely cited example of this is the soybean system (Weber et al 1974), in which the predominant isoenzyme (S-l), active at pH 9, has only scant ability to cooxidize pigments, whereas its so called acid isoenzymes (active at pH 6.5) cooxidize rapidly. Such differences between isoenzymes may be part of a larger pattern; wheat (McDonald 1976) and pea (Arens et al 1974) lipoxygenase isoenzymes also vary in pH optima and cooxidation potentials. Likewise, gel filtration of crude flax seed lipoxygenase preparations yields peaks of hydroperoxide-forming and bleaching activities that do not coincide (Weber et al 1973).

Hydroperoxide production and bleaching also differ in responses to various inhibitors. In the durum wheat system, in which pigment destruction during pasta production must be minimized (Irvine 1971), ascorbic acid will inhibit bleaching but not diene production (Walsh et al 1970). Ascorbic acid inhibition of lutein bleaching led McDonald (1979) to speculate that the highly reactive radical intermediates, generated in the lipoxygenase reaction, oxidized ascorbic acid in preference to the lutein pigment. Cysteine will selectively inhibit bleaching at high pH (McDonald 1976) and paradichloromecuriphenyl sulfonic acid will selectively enhance bleaching only at high pH.

Adding to the complexity of the problem, isoenzymes from several sources vary in the efficiency with which they bleach different pigments (Axelrod 1974). In what may be the most painstaking study reported, Yoon and Klein (1979) showed that the two main isoenzymes in pea differ radically in their ability to cooxidize β-carotene and chlorophyll; the rate of reaction with β-carotene as substrate for the cooxidation differed between isoenzymes; and finally, ratios of bleaching to peroxidation differed for each.

Grosch et al (1977) found that including the polyene crocin in lipoxygenase reaction mixtures alters the composition of the hydroperoxides formed. A higher percentage of the 13-L isomer is formed with the pigment present, indicating that the specificity of the peroxidative reaction is enhanced during bleaching.

Mechanism

Pigment destruction during lipid peroxidation is usually thought to stem from reactive intermediates generated in the peroxidative reaction (Grosch et al 1977, Veldink et al 1977, Yoon and Klein 1979), and the intermediates are thought to be free radicals. Free radicals in aerobic and anaerobic reaction paths are well known. The inability of preformed hydroperoxide plus enzyme alone to bleach and the ability for bleaching to occur anaerobically (Axelrod 1974) and in some cases, only anaerobically (Ikediobi 1977), adds support to the theory of a radical-mediated pathway. The ability to selectively inhibit bleaching while leaving conjugated diene production intact, coupled with an observed drop in carbonyl compound production (indicative of peroxide breakdown) while bleaching is going on, is cited as evidence that compounds produced before fatty acid hydroperoxide formation are the ones active in bleaching.

Most models for pigment bleaching involve the dissociation of the enzyme radical complex. Both Weber et al (1974) and Grosch et al (1977) favor the lipid peroxide (LOO·) as the reactive species in bleaching. Grosch et al (1977) hypothesize that competition between the polyene crocin and free linoleic acid for the 13-L hydroperoxide explains differences in 13 and 9 hydroperoxides formed in the presence of crocin (Fig. 4). A second model (Veldink et al 1977) suggests that dissociation produces the unoxygenated fatty acid radical, which is then free to attack pigment. Reference to DeGroot's model demonstrates at least one site in both the aerobic and anaerobic cycles at which such radicals could exist. In addition, differences among isoenzymes in strength of associative bonds between enzyme and radical might explain the observed differences in cooxidizing potentials. Possibly, the rate that various enzymes oxygenate the fatty acid radical could account for the differences observed.

ROLE OF LIPOXYGENASE IN BREADMAKING

The use of lipoxygenase in bread production can be divided into two broad categories. The first area, altering dough or bread color by coupled oxidation, is the oldest reported use for the enzyme (Haas and Bohn 1934). The second category is increasing mixing tolerance. Dough supplementation with enzymatically active soy flour has enjoyed considerable use in commercial production of white bread (Matz 1972, Ponte 1971).

Mixing Tolerance

Mixing tolerance is an important concept in the commercial production of bread. Commercially, mixing tolerance refers to the

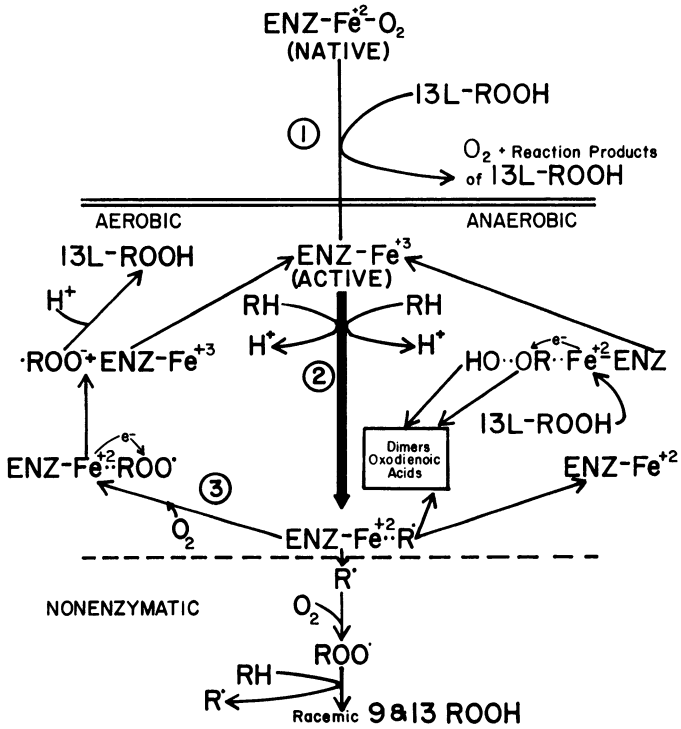

Fig. 3. DeGroot model for the aerobic and anaerobic reactions of soy lipoxygenase isoenzyme-1. **13-L-ROOH** = stereospecific hydroperoxide produced by the enzyme. (Adapted from DeGroot et al 1975.)

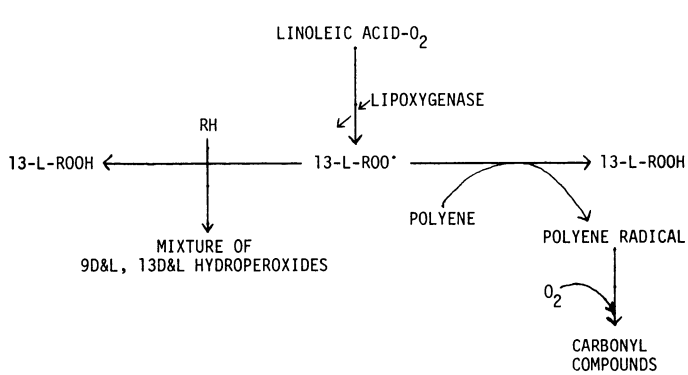

Fig. 4. Competition between linoleic acid **(RH)** and the polyene crocin for the 13-L hydroperoxide formed by lipoxygenase. Interaction with crocin results in its bleaching. (Adapted from Grosch et al 1977.)

ability of a dough to resist overmixing and subsequent breakdown after reaching peak development. In the laboratory, mixing tolerance can be accurately assessed with a mixograph (Fig. 5). Dough breakdown is seen as a decrease in the height and width of the mixogram tail after optimum mixing is reached. Tolerance can, therefore, be defined as a decrease in the rate of those changes.

Using the mixograph, Hoseney and his co-workers (Schroeder and Hoseney 1978, Weak et al 1977) determined effects of several compounds of mixing tolerance. Both fast-acting oxidants (KIO_3) and compounds containing activated double bonds, such as fumaric acid, decrease mixing tolerance. Addition of lipoxygenase (in the form of enzyme-active soy flour) increased mixing tolerance, thus giving graphic proof of a phenomenon long appreciated in the industry (Barrett 1975, Matz 1972). Lipoxygenase will reverse the effect of either KIO_3 (Fig. 5) or fumaric acid (Hoseney et al 1980). This was true in a nitrogen or an oxygen atmosphere, so lipoxygenase does not require oxygen to increase mixing tolerance.

In a study designed to discover how activated double bonds decrease dough stability, Sidhu et al (1980) found that fumaric acid will add to the thiyl radical created by the rupture of disulfide bonds during mixing. Grafting a bulky or charged species to the protein interferes with dough stability. Based on this, one can argue that activated double-bond compounds native to flour also would add to the free radical and, thereby, be responsible for dough breakdown from overmixing. Like lipoxygenase, certain free radical scavengers (eg, Tenox, a mixture of 20% butylated hydroxyanisole, 20% butylated hydroxytoluene, and 60% corn oil) will reverse dough breakdown caused by fumaric acid (Hoseney et al 1980).

Taken together, and taking into account lipoxygenase's ability to create fatty acid free radicals, the data suggest that Tenox or lipoxygenase increase mixing tolerance by stopping the addition reaction on the gluten protein. Adding exogenous activated double bond compounds accelerates the reaction by providing more reactant. Free radical scavengers such as butylated hydroxyanisole or butylated hydroxytoluene will quench the free radical and thus stop the addition. When lipoxygenase is added, it creates fatty acid radicals, which can then compete for any activated double-bond compounds. Lipoxygenase will, in fact, interfere with fumaric acid addition to gluten during mixing (Sidhu et al 1980).

Lipid Binding

The lipids in wheat flour and their role in breadmaking having been extensively reviewed (Chung et al 1978; Morrison 1978a, 1978b), making abundantly clear the fact that lipids present in flour and lipid composition affect the characteristics of the bread produced. In addition to their functionality, the extractability of flour lipids changes during the creation of dough. As moisture is added to wheat flour, a fraction of the free lipids becomes bound to protein and is no longer extractable with petroleum ether (Olcott and Mecham 1947). This phenomenon does not require mixing, but as mixing in air occurs, extractable lipid increases (Davies et al 1969).

Mixing is not the only factor that affects lipid extractability from dough. The presence or absence of oxygen also causes changes (Daniels et al 1966, 1969). The apparent difference is (Daniels et al 1970) that the percentage of lipids bound decreases in doughs mixed in oxygen but increases in doughs mixed without oxygen (N_2 atmosphere). As little as 1% oxygen in the mixing chamber was enough to detectably decrease lipid binding. Daniels and his co-workers have attributed the above phenomenon to lipid peroxidation during mixing (Daniels et al 1970, Frazer et al 1977). The effect of lipoxygenase on lipid binding has been recently reviewed (Frazier 1979).

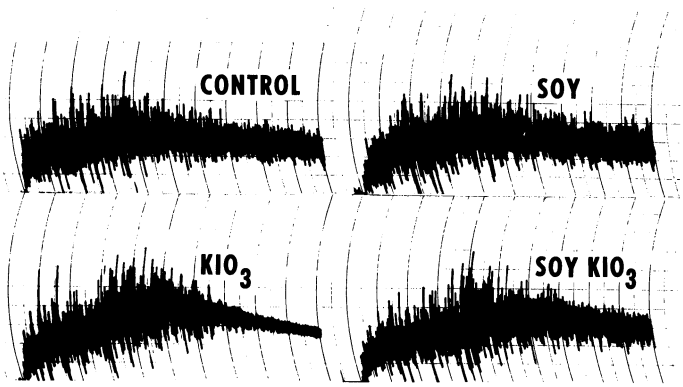

Fig. 5. Ten-gram mixograms demonstrating mixing tolerance and lack of tolerance. In the presence of KIO_3 **(lower left)** the curve breaks down rapidly. Addition of enzymatically active soyflower **(lower right)** restores mixing tolerance. The doughs were mixed under nitrogen. (Adapted from Hoseney et al 1980.)

Changes in Dough Rheology

In the production of bread and rolls of all types, addition of compounds known as improvers or oxidative improvers is common practice. Among these are the fast-acting oxidants KIO_3 and azodicarbonamide and the slow-acting oxidants $KBrO_3$ and ascorbic acid. The effect of these improvers is to change the dough rheology, to alter its physical-mechanical response to externally applied stress.

The lipoxygenase-linoleate system can be considered an improver in dough (Daniels et al 1974, Hoseney et al 1980, Kieffer and Grosch 1980). Using an Instron testing machine to measure "relaxation times" for doughs mixed to various levels of work imput, Frazier et al (1977) concluded that relaxation time increased with added soy lipoxygenase and that the increase was consistent with oxidative improvement of the dough. The effect required both free lipid and oxygen. Hoseney et al (1980) confirmed those results with a spread test (Hoseney et al 1979) that measures the spread of a molded dough piece under unit gravity.

Frazier et al (1977) further reported that heat inactivation of the enzyme negated its effects. Addition of preformed lipid peroxides (formed by either autoxidation of enzymatic action) had no effect on dough rheology. When the antioxidant nordihydroguaretic acid was added to dough, rheological effects were not impaired but peroxide formation was severely depressed. From this, Frazier et al (1977) concluded that lipid peroxides are not the active agents in the reaction and that a coupled oxidation mechanism is involved. The model takes as its basis the long-held view that soy flour imparts its improving effect in doughs by oxidizing sulfhydryls in gluten protein. This model is, in turn, based on a decrease in flour sulfhydryl groups seen when lipoxygenase was added (Tsen and Hlynka 1962, 1963). Although that work showed a correlation between lipoxygenase presence and sulfhydryl absence, it does not establish that the lipoxygenase-linoleate system was involved in the direct oxidation of these groups or establish the nature of the oxidized product (ie, whether it is now disulfide). Also, dismissal of lipid peroxides as active agents in lipoxygenase-mediated rheological changes ignores the presence of several highly reactive intermediates in the lipoxygenase reaction path. The ability of these compounds to react with a variety of proteins and amino acids (Gardner 1979, Karel et al 1975, Little and O'Brien 1968) makes the disulfide sulfhydryl interchange concept appear to be a severe overgeneralization. The conclusion can only be that no model yet exists to explain the effect of lipoxygenase on wheat flour dough rheology.

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