PHYSICOCHEMICAL CHARACTERIZATION OF BARLEY LIPOXYGENASE[1]

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ABSTRACT

Linoleic acid was used as the substrate for optimizing the assay of barley lipoxygenase in the cultivar Larker. Maximal activity was observed at a linoleic acid concentration of $5.47 \times 10^{-5} M$. The following parameters were determined: a) pH optimum, 6.0; b) temperature optimum, 47°C; c) average apparent K_m, $2.57 \times 10^{-6} M$; d) average V_{max}, 1.95×10^{-2} μmol/min; and e) activation energy, 2.2 kcal/mol to oxidize linoleic acid between 25° and 45°C. The enzyme was thermally stable up to 50°C and inactivated totally at 65°C. Compared to the activity produced by $5.47 \times 10^{-5} M$ linoleic acid at pH 6.0, linolenic acid and trilinolein were less reactive. Barley lipoxygenase was not inhibited by iron binding compounds. Inhibition by *p*-chloromercuribenzoate was irreversible. No activation was observed with Ca^{2+} or Mg^{2+}. The presence of one lipoxygenase-active electrophoretic band was detected by a lipoxygenase-specific staining procedure. At least 92% of the lipoxygenase activity is localized in the germ.

There are numerous investigations of stale flavor development through oxidative processes. However, little has been reported on the precise nature of oxidation in beer. It is known that the redox potential of beer decreases during storage, headspace oxygen decreases with time, and flavor deterioration is proportional to the amount of oxygen in the headspace (1). Mechanisms proposed for the oxidative production of stale flavor compounds include polyphenol (2,3) and melanoidin (4) mediated oxidation of precursor carbonyl compounds, oxidation of specific volatile phenolics (5), free radical autoxidation of long-chain unsaturated fatty acid moieties (6,7), melanoidin oxidation of higher alcohols (8), and the production of unusual volatile melanoidins (9). The catalytic influence of metals on oxidation of beer is documented (5,10).

The direct and indirect influence of polyphenols on the formation of volatile compounds present in stale beer has received the most attention. However, studies involving fatty acid oxidation (7,10) indicate that the compound primarily responsible for the cardboard flavor of stale beer is derived from a fatty acid precursor, as evidenced by the 60 to 80% decrease in C_{18} unsaturated fatty acids in beer during storage (11). Prevention of stale flavors will depend upon understanding the overall mechanism of staling.

If the oxidation of unsaturated fatty acids plays a role in the development of stale flavors in beer, the lipoxygenase system of barley and malt must be defined. The precise metabolic roles of barley and malt lipoxygenase are unclear. Barley lipoxygenase levels are increased during malting (12). Since the extent of lipoxygenase-mediated oxidation during malting and mashing is based on the lipoxygenase system of barley, the present study was undertaken to more precisely characterize the enzyme.

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MATERIALS AND METHODS

General Methods

All deoxygenated solutions were prepared by bubbling dry nitrogen gas into each for 15 min. All enzymatic activities were measured on a Beckman DB-G spectrophotometer equipped with a constant temperature cell and recorded continuously with a Beckman Laboratory Potentiometric recorder. All activity assays were performed in triplicate. Activity blanks were prepared by replacing the aliquot of the particular enzyme extract with the same volume of extraction buffer. Aliquots (10 ml) of the linoleic acid substrate and electrophoresis preparations and of the linolenic acid substrate preparation were frozen under an atmosphere of nitrogen until used. Trilinolein substrate preparations were stored at −10°C under a nitrogen atmosphere until needed. Similarly, substrate preparations were thawed (warmed, in the case of trilinolein) in a nitrogen atmosphere. All substrates were prepared in a nitrogen atmosphere. They were supplied by Applied Science Laboratories, Inc., State College, PA 16801, and were 99^{+}% pure.

Preparation of Barley and Kernel Fractions

Barley (*Hordeum vulgare* L.). The cultivar Larker, used throughout this study, was grown in a drill strip at Fargo, N. Dak., in 1972. Lightweight dockage was removed from harvested material with a Clipper fan mill (A. T. Ferrell and Co., Saginaw, MI 48605) equipped with sieves No. 7 and 13. The fan vent was approximately 3/4 open. Thin kernels and small, dense matter were removed from this partially cleaned material with a slotted 4.5/64-in. sieve. Any remaining foreign material and broken kernels were removed by hand. The cleaned barley was refrigerated until required.

Kernel fractions. Tissue fractions were prepared from 50-g lots by pearling with a Strong-Scott barley pearler (Strong-Scott Mfg. Co., Minneapolis, MN 55413). Each fraction was refrigerated until used.

An endosperm fraction was obtained by pearling for 2.75 min. This fraction was free of aleurone tissue except in the crease. Traces of scutellar tissue were probably present.

A germ fraction was prepared from dehusked barley. Husks were removed by initially pearling the barley for 4 sec. The resultant powder obtained by a second pearling of 1.75 min was purified by sieving with U.S. Standard Sieves (Tyler). Material passing a No. 10 sieve and retained by a No. 20 sieve was handpicked to remove all nongerm material. This fraction was free of endosperm and aleurone tissues.

Since recovery of germ was not quantitative, distal and proximal ends were prepared by laterally transecting whole kernels with a kernel cutter designed to halve 50 kernels by one movement of its blade. The kernel ends were separated and refrigerated until needed.

Grist. A portion of the barley was ground finely with a Miag malt mill (Buhler-Miag, Inc., Minneapolis, MN 55426), standardized according to the ASBC approved method (13). Some of the endosperm tissue and separated kernel ends were ground in the same manner. All grists were refrigerated until extracted.

Lipoxygenase

Extraction. Lipoxygenase extracts were prepared daily prior to measurement.

Extractions were performed with cold (3°−5° C) 0.05*M* phosphate buffer, pH 6.0, in an ice bath for 1 hr, swirling 30 sec every 15 min. Finely ground whole kernels and endosperm grists were extracted with 10 vol of buffer, while finely ground distal and proximal ends were extracted with 20 vol. Germ tissue was frozen in liquid nitrogen and crushed with a mortar and pestle prior to extraction with 50 vol of buffer. After it was centrifuged at 15,000 × *g* for 15 min at 4° C, the resultant supernatant was poured through glass wool into a graduated cylinder, and the volume recorded.

Substrates. Linoleic acid substrate was prepared by a modified method of Surrey (14). Linoleic acid (0.25 ml) was added dropwise to a solution of 5.0 ml of deoxygenated 0.05*M* borate buffer, pH 9.0, containing 0.25 ml of Tween-20 (Fisher Scientific Co., Fair Lawn, NJ 07410), with continuous stirring. The resultant dispersion was clarified by adding 0.65 ml of 1*N* sodium hydroxide. The volume was adjusted to 50 ml with additional deoxygenated borate buffer and then to 100 ml with deoxygenated distilled water. The final concentration of this substrate solution was $8.05 \times 10^{-3} M$.

Linolenic acid substrate was prepared by the same general procedure used for linoleic acid, except that all volumes were halved. The final concentration was $8.22 \times 10^{-3} M$.

Trilinolein (100 mg) was stirred into a solution of 5.0 ml of 0.05*M* deoxygenated borate buffer, pH 9.0, and 0.125 ml of Tween-20. After addition of 0.33 ml of 1*N* sodium hydroxide, complete dispersion was achieved by adjusting the final volume to 50 ml with deoxygenated ethanol. The final concentration was $2.20 \times 10^{-3} M$.

Assay procedure. Lipoxygenase activity was determined by measuring the increase in absorbance at 234 nm at 25° C (15). Activity was recorded for 3 min, and the increase in absorbance during the first minute was used for all calculated activities. In a typical assay mixture, 2.90 ml of 0.05*M* phosphate buffer, pH 6.0, and 0.02 ml of the recently prepared extract were mixed in a cuvet, and 0.02 ml of the recently thawed or warmed substrate solution was added. One unit of activity is equivalent to an increase in absorbance of one at 234 nm at 25° C.

Prior to assaying, the level of substrate autoxidation was determined by the method of MacGee (16). Substrate was discarded when its conjugated diene concentration was 3% or higher, as determined by the absorbance at 234 nm of 0.10 ml of substrate plus 2.90 ml of deoxygenated distilled water against a distilled water blank.

Optimum temperature. The assay buffer was preheated to the temperature at which measurements were performed, while the enzyme extract and linoleic acid substrate were kept on ice. A cuvet containing 2.90 ml of assay buffer plus 0.02 ml of enzyme extract was placed in the spectrophotometer and maintained for 1.0 min at the desired temperature to ensure equilibration. Following addition of 0.02 ml of the linoleic acid substrate, activity at 234 nm was recorded.

Thermal stability. This parameter was determined by incubating 5.0 ml of the ice-cold enzyme extract in a water bath at the desired temperature for 5 min. The incubated extract was cooled on ice and assayed at 25° C according to the previously described procedure.

Inhibitors. All inhibitors tested were analytical grade.

Gel electrophoresis. Disc polyacrylamide gel electrophoresis was performed with the Polyanalyst analytical polyacrylamide vertical gel electrophoresis

apparatus (Buchler Instruments, Fort Lee, NJ 07024), using the anionic gel system (running gel, pH 9.3) detailed in the accompanying manual. The running gel was modified in that it contained 0.5% soluble starch that served as an indicator for the staining procedure (17).

The developed gel was incubated for 30-45 min in test tubes containing a linoleic acid solution specially prepared for this application. The incubated gel was immediately immersed in a freshly prepared 15% (v/v) aqueous acetic acid solution containing 5% (v/v) saturated potassium iodide. The gel was soaked until color development was maximized without excessive background stain.

The linoleic acid preparation used for electrophoresis was prepared by adding 0.08 ml of linoleic acid dropwise to 10.0 ml of deoxygenated 0.05 *M* phosphate buffer, pH 6.0, containing 0.25 ml of Tween-20 under continuous stirring. The dispersion was clarified by adding 0.65 ml of 1 *N* sodium hydroxide. The volume was adjusted to 100 ml with additional deoxygenated buffer. The final concentration of this preparation was 2.50×10^{-3} *M*.

Kinetic constants. The apparent Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) for linoleic acid were calculated with linear regression equations, treated according to the Lineweaver-Burk (1/v *vs.* 1/[S]), Eadie-Hofstee (v *vs.* v/[S]) and Hanes ([S]/v *vs.* [S]) methods (18). A molar extinction coefficient of 2.80×10^4 was used in all calculations (19). In the 2.94-ml assay mixture, an increase in absorbance of one equaled the oxidation of 0.11 μmol of linoleic acid.

The activation energy required to oxidize linoleic acid between 25° and 45°C was determined with a linear regression equation. The results were analyzed by an Arrhenius plot (log v *vs.* $1/°K \times 10^3$), using the slope to calculate the activation energy.

Hydroperoxide Isomerase

Extraction. Hydroperoxide isomerase extracts were obtained by the procedure used to prepare lipoxygenase extracts.

Substrate. The hydroperoxide substrate (15) was prepared by incubating 0.2 ml of the linoleic acid substrate solution and 0.4 ml of a stock soybean lipoxygenase solution (Sigma Chemical Co., St. Louis, MO 63178) in 10.0 ml of distilled water for 15 min. Stock soybean lipoxygenase (type II) was prepared at a concentration of 1 mg/ml with 0.05 *M* borate buffer, pH 9.0. This substrate was always prepared the day the assay was performed.

Assay procedure. Hydroperoxide isomerase activity was determined by measuring the decrease in absorbance at 234 nm at 25°C (15). The decrease in absorbance during the first minute of assaying was used for all calculated activities. The assay mixture contained 2.45 ml of 0.05 *M* phosphate buffer at pH 6.0, 0.53 ml of freshly prepared hydroperoxide substrate, and 0.02 ml of freshly prepared extract. One unit of activity is equivalent to a decrease in absorbance of one at 234 nm at 25°C.

Protein Determination

The soluble protein content of each extract was determined at 540 nm by the biuret method of Gornall *et al.* (20). Absorbance was converted to protein concentration by reference to a standard curve prepared with varying concentrations of bovine serum albumin (Sigma Chemical Co., St. Louis, MO 63178).

RESULTS AND DISCUSSION

Lipoxygenase

pH. The profile of barley lipoxygenase activity with pH is shown in Fig. 1. The pH optimum of 5.9 to 6.0 was lower than the pH optimum of 6.8 reported by Franke and Frehse (21). The difference between these pH optima is probably due to the presence of Tween-20 in the assay medium. Tween-20 may influence the recognized micellar effects on activity (22,23) and thus lower the pH optimum by preventing precipitation of the protonated form of linoleic acid substrate present at acidic pH values.

The increase in activity at pH 9.0 may be the result of a change in micellar effects or the manifestation of some isozymic effects. The general shape of the pH activity curve, with optimum activity at pH 6.0 and a second activity rise at pH 9.0, is very similar to that determined for soybean lipoxygenase (24). However, the activity plateau between pH 4.0 and 5.0 was not observed with soybean lipoxygenase (24), nor was it seen in the early work done with barley lipoxygenase (21).

Temperature. Thermal stability studies on barley lipoxygenase revealed no appreciable differences in activity at incubation temperatures of 0° to 50°C. Activity steadily declined at higher temperatures, with total inactivation occurring at 65°C. The optimum temperature of barley lipoxygenase was 47°C. Since these parameters are routinely determined, the data are not presented.

Substrate concentration. The effect of substrate concentration on mung bean lipoxygenase activity was noted by Surrey (14), who observed optimal activity with 0.25% linoleic acid and decreased activity with higher concentrations. Consequently, the optimum concentration of the linoleic acid substrate was determined for barley lipoxygenase. The results are shown in Fig. 2. Optimum activity was attained when the total incubation volume of 2.94 ml contained 0.02 ml of the $8.05 \times 10^{-3} M$ linoleic acid substrate solution. Thus, optimum activity was achieved at a linoleic acid concentration of $5.47 \times 10^{-5} M$. This concentration is nearly 100 times less than that used by Franke and Frehse (21).

Substrate reactivity. In addition to linoleic acid, linolenic acid and trilinolein were tested as substrates because of their reactivity with lipoxygenases from

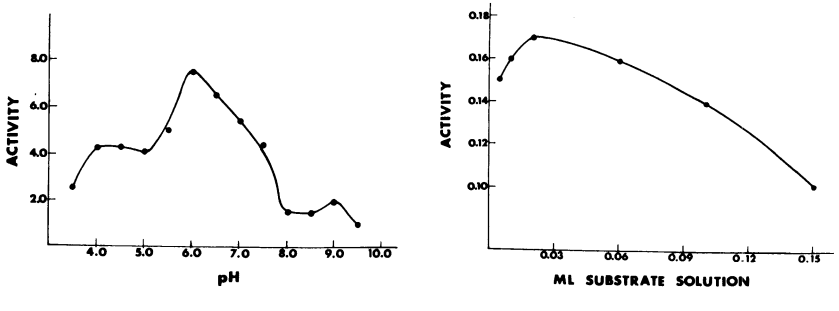

Fig. 1. Profile of barley lipoxygenase activity (ΔA_{234}/min/ml) with pH, determined at a linoleic acid concentration of $5.47 \times 10^{-5} M$. Fig. 2. Effect of substrate concentration (ml of the $8.05 \times 10^{-3} M$ linoleic acid substrate solution/2.94 ml of assay mixture) on barley lipoxygenase activity (ΔA_{234}/min/0.02 ml).

other sources (25). Activity measured with each substrate was determined under conditions used for linoleic acid (pH 6.0, 25° C, and 0.02 ml of substrate). Under these conditions, barley lipoxygenase exhibited 6.00 units of activity with linolenic acid and 1.00 unit of activity with trilinolein, compared to 8.00 units of activity with linoleic acid.

The lower reactivities observed with linolenic acid and trilinolein may be due to micellar effects and their influence on activity (22,23). Since critical micelle concentrations were not determined, it can be concluded that, under the conditions of measurement, each serves as a substrate for barley lipoxygenase. Conclusions involving specificity should not be drawn when various substrates are employed, as differences in micellar characteristics are most likely operative.

Inhibitors. Barley lipoxygenase activity was measured in the presence of several well-recognized enzyme inhibitors among which were ions, chelators, and thiol-specific reagents. The results are listed in Table I.

Inhibition of barley lipoxygenase by compounds specific for sulfhydryl groups was unexpected, because wheat lipoxygenase is not inhibited by sulfhydryl binding reagents like *p*-chloromercuribenzoate (25). This inhibition by sulfhydryl blocking reagents was not reversed by addition of a 100 *M* excess of the reducing agent dithioerythritol. On the other hand, mung and urd bean lipoxygenases are inhibited by *p*-chloromercuribenzoate and partially reactivated after addition of excess reducing agent (26). Barley lipoxygenase, like lipoxygenases from other sources (27), is not inhibited by cyanide, azide, or EDTA. This observation is supported by the recent discovery that the iron of soybean lipoxygenase is not bound by 1,10-phenanthroline until the enzyme has been treated with a reducing agent (28–30).

Barley lipoxygenase activity was neither inhibited nor activated by magnesium or calcium cations (Table I), although other studies have shown calcium to be an activator (21). This observed lack of calcium activation is ascribed to the recognized micellar effect produced by the presence of Tween-20 (23). Inhibition

TABLE I
Effect of Inhibitors on Barley Lipoxygenase Activity

Effector[a]	Inhibition[b] %
p-Chloromercuribenzoate	100
Hg++ (Mercuric chloride)	93
N-Ethylmaleimide	87
Cu++ (Cupric sulfate)	100
Dithiothreitol	0
Cysteine	0
CN^- (Sodium cyanide)	0
N_3^- (Sodium azide)	0
EDTA[c]	0
Mg++ (Magnesium chloride)	0
Ca++ (Calcium chloride)	0
Ascorbic acid	89

[a]Each effector concentration was 1 m*M*, except cysteine at 2 m*M*.
[b]Those effectors showing 0% inhibition also showed no activation.
[c]Disodium salt of (ethylenedinitrilo)-tetra-acetic acid.

by ascorbic acid was anticipated, as it is a competitive inhibitor of durum wheat lipoxygenase (31).

Electrophoresis. Because high levels of lipoxygenase activity are required for the electrophoretic method of Guss *et al.* (17), aliquots containing 3.5 to 17.5 units of lipoxygenase activity were used for isozyme determinations. In all cases, whether a crude preparation or an ammonium sulfate fraction was examined, only one electrophoretic band was observed. This was unexpected because four lipoxygenase-active isozymes have been detected in soybean, pea, and wheat (17,32). If isozymic forms are present in barley, a more sensitive assay is required to reveal them.

Kinetic constants. When the calculated slope and y-intercept values were inserted into the general equation of each linear treatment of the Michaelis-Menten equation, apparent K_m's of $2.44 \times 10^{-6} M$ (Lineweaver-Burk), $2.47 \times 10^{-6} M$ (Eadie-Hofstee), and $2.80 \times 10^{-6} M$ (Hanes) were calculated. From these values, an average apparent K_m of $2.57 \times 10^{-6} M$ was determined. An average V_{max} of 1.95×10^{-2} μmol/min was calculated in a similar fashion. The data used to calculate the average K_m and V_{max} constants are shown in Table II.

An apparent K_m of $1 \times 10^{-3} M$ was reported (21) for barley lipoxygenase with the less sensitive oxygen-uptake assay procedure and substrate concentrations averaging 100 to 1000 times higher than those of this study. The area of substrate concentration is a most important consideration, as high linoleic acid concentrations inhibit barley (Fig. 2) and mung bean (14) lipoxygenases. In addition, the apparent K_m for durum wheat lipoxygenase of $5 \times 10^{-6} M$ was determined manometrically with a linoleic acid substrate stabilized with a detergent (31). Therefore, the average apparent K_m reported here is probably more accurate than that determined earlier for barley lipoxygenase (21).

An activation energy of 2.2 kcal/mol was calculated for barley lipoxygenase within the inclusive range of 25° to 45°C. An activation energy of barley lipoxygenase was not determined by Franke and Frehse (21), as constant initial velocities at 20°, 30°, and 40°C were reported. However, the activation energy determined in this study is similar to the 2.5 kcal/mol determined for durum wheat lipoxygenase at temperatures over 18°C (33).

TABLE II
Data Used to Calculate Barley Lipoxygenase K_m and V_{max}

	Assay Number[a]		
	1	2	3
Volume of linoleic acid (μl)[b]	5	10	20
Linoleic acid in assay mixture (μM)	1.37×10^{1}	2.74×10^{1}	5.47×10^{1}
v (ΔA_{234}/min)	1.50×10^{-1}	1.60×10^{-1}	1.70×10^{-1}
v ($\Delta\mu$mol/min)	1.65×10^{-2}	1.76×10^{-2}	1.87×10^{-2}
1/v (min/μmol)	6.06×10^{1}	5.68×10^{1}	5.35×10^{1}
1/[S] (1/μM)	7.30×10^{-2}	3.65×10^{-2}	1.83×10^{-2}
[S]/v (min μM/μmol)	0.83×10^{3}	1.56×10^{3}	2.93×10^{3}
v/[S] (μmol/min μM)	1.21×10^{-3}	0.64×10^{-3}	0.34×10^{-3}

[a]Activity at each substrate level was determined in triplicate.
[b]Concentration of linoleic acid preparation was $8.05 \times 10^{-3} M$.

Hydroperoxide Isomerase

Hydroperoxide isomerase is the recently reported enzyme that uses the lipoxygenase-derived conjugated diene hydroperoxide as its substrate (15). This activity was observed in this study when lipoxygenase extracts were assayed for longer than 3 min. After 3 min, a continuous decrease in absorbance at 234 nm was observed. Preferential measurement of lipoxygenase activity in phosphate buffer was achieved by using 0.05*M* tris-hydrochloride buffer, pH 7.0, for extraction. On the other hand, isomerase activity was not selectively inhibited if phosphate buffer extracts were assayed in tris-buffer. In addition, fractionation of a tris-buffer extract with 40 to 60% ammonium sulfate readily reversed isomerase inhibition, as both activities were observed in all fractionated tris-buffer extracts. However, since assay times greater than 3 min were required to observe isomerase activity, extraction was done in phosphate buffer because the resultant lipoxygenase activity was higher.

When hydroperoxide isomerase activity was measured specifically, the procedure described for this assay was used. There is no interference from lipoxygenase during assay, as the conjugated diene hydroperoxide is the substrate of this reaction (15).

Localization of Lipoxygenase and Hydroperoxide Isomerase Activities within the Barley Kernel

The distribution of lipoxygenase and hydroperoxide isomerase activities within the barley kernel is listed in Table III.

Minor levels of lipoxygenase and hydroperoxide isomerase activities are located in the endosperm, whereas high levels of both activities are associated with the germ. If the levels of each activity measured in the proximal and distal ends are summed, 99% of the lipoxygenase activity and 84% of the hydroperoxide isomerase activity are concentrated in the germ end. The specific activity of each enzyme associated with the endosperm is identical to that localized in the distal end. Since distal ends possess husk and aleurone tissues, these two tissues possess low levels or are devoid of lipoxygenase and isomerase activities. The minor activities present in the distal end are probably localized in the endosperm.

When the total lipoxygenase activities present in whole barley and the proximal end are compared, only 92% of the total activity present in whole barley

TABLE III
Distribution of Lipoxygenase and Hydroperoxide Isomerase Activities within the Barley Kernel

	Lipoxygenase		Hydroperoxide Isomerase	
Fraction	Total[a]	S.A.[b]	Total[a]	S.A.[b]
Whole barley	79.95	5.93	4.00	0.37
Distal end	0.45	0.10	0.90	0.19
Proximal end	73.75	9.74	4.80	0.73
Endosperm	...[c]	0.10	...[c]	0.19
Germ fragments	...[c]	19.16	...[c]	1.64

[a]Units of activity/g of seed.
[b]Specific activity; units of activity/mg of soluble protein.
[c]Recovery of fraction was not quantitative.

is associated with the proximal kernel end (Table III). This level of total activity is 7% less than that associated with the proximal end on the basis of the summed activity present in the proximal and distal kernel ends. This difference is attributed to one or all of the following: a) the possible concentration of hydroperoxide isomerase activity during transection and concomitant increased chromophore destruction, b) the possible removal of a lipoxygenase activator during transection, or c) the possibility that the transected kernels contained more tissue than that strictly associated with the proximal end, whereby both germ tissue and its associated lipoxygenase activity were diluted.

In summary, barley lipoxygenase exhibits a high affinity for linoleic acid (low K_m), optimal activity at low concentrations of linoleic acid, and a low activation energy. In addition, the trihydroxy-octadecenoic acids detected in finished beer (7) and produced from linoleic acid by barley lipoxygenase suspensions (34) are associated with stale beer flavors. The 169% increase in lipoxygenase activity during malting (12) is consistent with the involvement of lipoxygenase in the development of stale flavors in beer.

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Literature Cited

1. DeCLERCK, J. Applications in brewing. J. Inst. Brew. 40: 407 (1934).
2. LINDSAY, R. C. Stale flavors in beer. Tech. Quart. Master Brew. Ass. Amer. 10: 16 (1973).
3. OWADES, J. L., and JAKOVAC, J. Study of beer oxidation with O^{18}. Amer. Soc. Brew. Chem., Proc. 1966, p. 180.
4. BURGER, M., GLENISTER, P. R., and BECKER, K. Acrolein and other aldehydes in beer. Amer. Soc. Brew. Chem., Proc. 1954, p. 98.
5. DADIC, M. Phenolics and beer staling. Tech. Quart. Master Brew. Ass. Amer. 11: 140 (1974).
6. DOMINGUEZ, X. A., and CANALES, A. M. Oxidation of beer. A rational mechanism for the degradation of unsaturated fatty acids and the formation of unsaturated aldehydes. Brew. Dig. 49(7): 40 (1974).
7. DROST, B. W., van EERDE, P., HOEKSTRA, S. F., and STRATING, J. Fatty acids and staling of beer. Eur. Brew. Conv., Proc. Congr. 13th, Estoril, 1971, p. 451.
8. HASHIMOTO, N. Oxidation of higher alcohols by melanoidins in beer. J. Inst. Brew. 78: 43 (1972).
9. FERRETTI, A., and FLANAGAN, V. P. The lactose-casein (Maillard) browning system: Volatile components. J. Agr. Food Chem. 19: 245 (1971).
10. MEILGAARD, M. Stale flavor carbonyls in brewing. Brew. Dig. 47(4): 48 (1972).
11. VISSER, M. K., and LINDSAY, R. C. Ester precursors of aldehydes associated with staling of beer. Tech. Quart. Master Brew. Ass. Amer. 8: 123 (1971).
12. LULAI, E. C., and BAKER, C. W. The alteration and distribution of lipoxygenase in malting barley and in finished malt. Proc. Amer. Soc. Brew. Chem. 33(4): 154 (1975).
13. AMERICAN SOCIETY OF BREWING CHEMISTS. Methods of analysis, Malt-4. The Society: St. Paul, Minn. (1958).
14. SURREY, K. Spectrophotometric method for determination of lipoxygenase activity. Plant Physiol. 39: 65 (1964).
15. ZIMMERMAN, D. C., and VICK, B. A. Hydroperoxide isomerase: A new enzyme of lipid metabolism. Plant Physiol. 46: 445 (1970).
16. MacGEE, J. Enzymatic determination of polyunsaturated fatty acids. Anal. Chem. 31: 298 (1959).
17. GUSS, P. L., RICHARDSON, T., and STAHMANN, M. A. The oxidation-reduction enzymes of wheat. III. Isoenzymes of lipoxidase in wheat fractions and soybean. Cereal Chem. 44: 607 (1967).
18. DIXON, M., and WEBB, E. C. Enzymes (2nd ed.), pp. 63-70. Academic Press: New York (1964).

19. PRIVETT, O. S., NICKELL, C., and LUNDBERG, W. O. Products of the lipoxidase-catalyzed oxidation of sodium linoleate. J. Amer. Oil Chem. Soc. 32: 505 (1955).
20. GORNALL, A. G., BARDAWILL, C. S., and DAVID, M. M. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751 (1949).
21. FRANKE, W., and FREHSE, H. Autoxidation of unsaturated fatty acids. VI. On the lipoxidase in *graminae,* in particular barley. Hoppe-Seyler's Z. Physiol. Chem. 295: 333 (1953).
22. ALLEN, J. C. Soybean lipoxygenase. 1. Purification, and the effect of organic solvents upon the kinetics of the reaction. Eur. J. Biochem. 4: 201 (1968).
23. ZIMMERMAN, G. L., and SNYDER, H. E. Role of calcium in activating soybean lipoxygenase-2. J. Agr. Food Chem. 22: 802 (1974).
24. AMES, G. R., and KING, T. H. The assay and pH profile of lipoxidase. J. Sci. Food Agr. 17: 301 (1966).
25. TAPPEL, A. L. Lipoxidase, p. 275. In: The enzymes, ed. by P. D. Boyer, H. Lardy, and K. Myrback, Vol. 8. Academic Press: New York (1963).
26. SIDDIQI, A. M., and TAPPEL, A. L. Comparison of some lipoxidases and their mechanism of action. J. Amer. Oil Chem. Soc. 34: 529 (1957).
27. WHITAKER, J. R. Lipoxygenase, p. 607. In: Principles of enzymology for the food sciences, ed. by O. R. Fennema. Marcel Dekker, Inc.: New York (1972).
28. CHAN, H. W. S. Soya-bean lipoxygenase: An iron-containing dioxygenase. Biochim. Biophys. Acta 327: 32 (1973).
29. PISTORIUS, E. K., and AXELROD, B. Iron, an essential component in lipoxygenase. J. Biol. Chem. 249: 3183 (1974).
30. ROZA, M., and FRANCKE, A. Soybean lipoxygenase: An Iron-containing enzyme. Biochim. Biophys. Acta 327: 24 (1973).
31. IRVINE, G. N., and ANDERSON, J. A. Kinetic studies of the lipoxidase system of wheat. Cereal Chem. 30: 247 (1953).
32. HALE, S. A., RICHARDSON, T., VON ELBE, J. H., and HAGEDORN, D. J. Isozymes of lipoxidase. Lipids 4: 209 (1969).
33. IRVINE, G. N. Effect of temperature on the kinetics of the lipoxidase system of wheat. Cereal Chem. 36: 146 (1959).
34. GRAVELAND, A., PESMAN, L., and van EERDE, P. Enzymatic oxidation of linoleic acid in barley suspensions. Tech. Quart. Master Brew. Ass. Amer. 9: 98 (1972).

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