Influence of Barley and Malt Storage on Lipoxygenase Reaction

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ABSTRACT

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The dioxygenation of linoleic acid (LA) by aqueous flour suspensions of barley and malting samples was studied. The rate of this lipoxygenase (LOX) reaction varied as the malting process proceeded, giving a characteristic LOX reaction profile for a malting. The differences in the profiles from one malting to another were dramatic. It also appeared that during storage of dry, intact kernel samples from a single malting, a reduction in the rate of LOX reaction always occurred, and the rates of reduction with time were dependent on the stage of malting at the time of sampling. The kinetics of this aging could roughly be divided into four categories representing different stages of malting. Consequently, greatly varying LOX reaction profiles can be obtained from a single malting depending on the time of storage of kernels before assays. The results indicate that steeping, germination and the subsequent drying render the state of kernels unstable with respect to the LOX reaction for at least two to three weeks. Homogeneity of malt quality is important in the further applications of malt, especially in the brewing industry. Therefore, the rate of LOX reaction should be considered as a quality factor of malt.

Oxidation of lipids during processing or storage of cereals is a complicated phenomenon where the state of the substrate and the balance between antioxidative and prooxidative factors of the mixture play an important role. However, before the influence of oxidation on the quality of cereal products can be predicted, the amount of the oxidative enzymes in the cereal material as well as the ability to react on their respective substrates (i.e., actual activity under processing conditions in the mixture) has to be clarified. Regardless of the actual mechanism of the oxidation and the reasons for it, oxidation of lipids reduces the nutritional value of cereal products, affects the color, and the appearance of the products, and causes the formation of off-odors and off-flavors. Thus, the overall quality of cereal products is affected (Faubion and Hoseney 1981, Gardner 1989, Drost et al 1990, Kobayashi et al 1993). Lipoxygenase (LOX) or oxidized lipid components might also cause problems during the processing itself (Wheeler and Wallace 1978, Gardner 1989).

Oxidation of lipids can be enzymatic or nonenzymatic. The main enzyme that oxidizes cereal lipids is LOX (EC 1.13.11.12), a nonheme ferrous protein, that catalyzes oxidation of polyunsaturated fatty acids with a 1,4-*cis*-*cis*-pentadiene structure. The products of LOX reaction are unstable hydroperoxides that form small aldehydes and ketones in further reactions. One of these products is *trans*-2-nonenal that is believed to cause stale flavor in beer (Yang and Schwarz 1995).

Barley is reported to contain two LOX isoenzymes (Yabuuchi 1976, Baxter 1982, van Aarle et al 1991, Doderer et al 1992, Yang et al 1993). The isoenzyme LOX I occurs mainly in sound, ungerminated barley, whereas isoenzyme LOX II appears during germination (Yang and Schwarz 1995). The amount of LOX I and LOX II increases during malting of barley (Yang and Schwarz 1995). Also, the ratio between LOX I and LOX II changes during the malting process (Holtman et al 1996). In recent years, these barley LOX have been purified and well characterized (van Aarle et al 1991, Doderer et al 1992, Huques et al 1994).

On the other hand, the influence of LOX on lipids during processing of barley and malt is difficult to determine. Based on the available information, this problem has also been discussed by van Waesberghe (1997). Most of the difficulties are due to the nature of the natural substrate of LOX, linoleic acid (LA). The solubility of LA in water is poor and, even in model systems containing only LOX and LA, only a small part of the substrate is available for the enzyme. In addition, when components that can bind the substrate or change its solubility are present in the reaction mixture, the reaction becomes less dependent on the amount and activity of the enzyme and depends rather on the state and availability of the substrate. Therefore, the observed LOX activity is a net result of the enzyme activity and the physicochemical state of the substrate.

This article presents the influence of storage of barley, malt, and samples taken during malting on the LOX reaction in aqueous solutions of samples. The method used in this study to determinate the oxidation caused by LOX measures, in fact, the actual influence of the sample material and its components on availability of LA for LOX under reaction conditions. Three aspects are addressed: 1) the significance of the changes that occur during malting and the subsequent storage in cereal material on LOX reactions; 2) the significance of storage time and conditions on LOX reaction; and 3) the significance of the state and availability of substrate for LOX on the overall lipid oxidation.

MATERIALS AND METHODS

Materials

Finnish malting barley cultivars Kymppi (crop years 1993 and 1994) and Kustaa (crop year 1993) were used. Moisture content of the barley varieties was 12–13%. All other chemicals were of reagent grade or higher.

Methods

Experiments were perfromed using malts that were micromalted employing 44-hr steeping with varying wet and dry periods (6 hr wet, 13 hr dry, 5 hr wet,19 hr dry, and 1 hr wet) at 12–14°C, five-day germination at 14–16°C, and 22 hr of kilning. Samples were taken after each step during steeping and after every germination day. Malting samples were either freeze-dried or stored frozen at –20°C. Moisture content of the samples was <5% after freeze-drying.

The rate of CO_2 production was measured as described by Reinikainen et al (1996).

Barley and dried malting samples were stored altogether for 230 days at 5°C. Frozen samples were stored at –20°C for six months, freeze-dried, and stored after drying at 5°C for 190 days. For comparison, some of the samples were also stored both at room temperature and at 5°C for 120 days.

Assay of LOX Reaction

LOX reaction (i.e., dioxygenation of LA) was measured in an aqueous sample of flour suspension without extraction or purification of the enzyme. Rate of LOX reaction was determined as the rate of O_2 consumption with the addition of LA substrate using an oxygen monitor (YSI model 5300, Yellow Springs Instruments, Yellow Springs, OH). A 100-mg ground sample was suspended in 7 mL of 50 m*M* Na-phosphate buffer, pH 7.0, in a reaction cham-

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ber at 25°C. When balance was achieved, the oxidation reaction was initiated by the addition of 300 µL of LA suspension containing 0.86 g of free LA in Tween 20 and NaOH solution (Axelrod et al 1981). The LOX unit was defined as the quantity of enzyme that consumes 1 µmol of O_2/min under the assay conditions. The activities were calculated per dry weight of samples.

Measurements of LOX reaction at different pH levels were made as above but with reaction buffers of 50 m*M* Na-acetate at pH 4.5, 5.0, and 5.5; with 50 m*M* Na-phosphate buffers at pH 6.0, 6.5, 7.5, 8.0, and 8.5; and with 50 m*M* Na-borate buffer pH 9.0, 9.5, and 10.0.

The data on the rate of LOX reaction are mean values of at least two determinations. The maximum standard deviation for the rate of LOX reaction was 10%.

RESULTS

The LOX reaction measured directly in flour-water suspension varies significantly depending on the stage of malting process (Kaukovirta-Norja et al 1993, 1995). In the present study, the profile of LOX reaction in samples taken during malting was measured as in previous studies (Kaukovirta-Norja et al 1993, 1995) and resembled the earlier profile (Fig. 1). At the end of malting, in lager malt the rate of LOX reaction was 19% of the rate found in barley. Similarly, in studies of Tressl et al (1979), LOX activity was measured only at six different stages of malting, but the main trends resembled the trends obtained in the present study. However, even though the profile of LOX reaction during malting was similar to those of previous reports, the level of LOX reaction throughout the malting was different. The rates in suspensions of samples taken during steeping and germination were, in the present study, markedly faster than in the earlier study (Kaukovirta-Norja et al 1993). The poor repeatability of LOX reaction between samples taken at the same stage of malting but from different maltings has also been observed during previous studies. However, a rational explanation for this variation was not the usage of different cultivars, usage of cultivars from different crop years, nor the differences in the malting program. The only identifiable variable between experiments was the storage time of dried samples. Therefore, the rate of LOX reaction was measured after different storage periods.

Influence of Storage on LOX Reaction

The influence of storage on the chemical and physical composition of malt is not well documented. However, it is known that no significant changes in activities of amylases or proteinases occur during a limited storage time (e.g., six months). Instead, storage of malt is reported to improve the separation of wort (Rennie and Ball 1979), indicating that changes in the material have occurred. Therefore, it is probable that changes in the interactions of kernel material with LA or LOX, and consequently in the interaction between LA and LOX, also occur.

To elucidate the influence of sample age, dried samples were stored at 5°C over a seven-month period (230 days). LOX reaction was measured in fresh malting samples, samples stored 56 days, and samples stored 230 days. Figure 1 shows that storage reduced significantly the ability of samples to oxidize LA, indicating changes either in LOX activity of samples or in the other properties of the sample material. However, the effect of aging was not as marked in all samples. Figure 2 (a–f) presents six samples (barley, one-day germinated barley, five-day germinated barley, 3-hr kilned malt, and 12-hr kilned malt, and lager malt) in which LOX reaction showed different aging kinetics.

The malting process could be divided into four main categories in which the kinetics of aging was different. The first category includes barley alone (Fig. 2a). After almost seven months of storage, 83% of the original rate of LOX reaction was still present in barley. This is in accord with the study of Fretzdorff and Jördens (1986), who reported the presence of >80% of the LOX activity of barley after two and a half months of storage at 4°C. The second category includes samples taken during germination and during early hours of kilning (Fig. 2b–d). In this category, the rate of the LOX reaction was the most markedly reduced, and aging followed first-order kinetics. The LOX reaction was reduced significantly during the first 50 days, after which the reduction was moderate and linear. Only 16% of the original LOX reaction remained in the five-day germinated sample after 230 days of storage. The third category is represented by samples taken after the 12-hr kilning (Fig. 2e). The reduction in LOX reaction during the 230 days of storage was moderate (25–40%). Furthermore, the reduction with time was linear. The fourth category, lager malt (Fig. 2f), was considerably more stable with reference to the reduction in the LOX reaction during storage. Furthermore, the LOX reaction in lager malt was reduced linearly as a function of time (Fig. 2f). More than 75% of the rate of LOX reaction was found in lager malt after 230 days of storage.

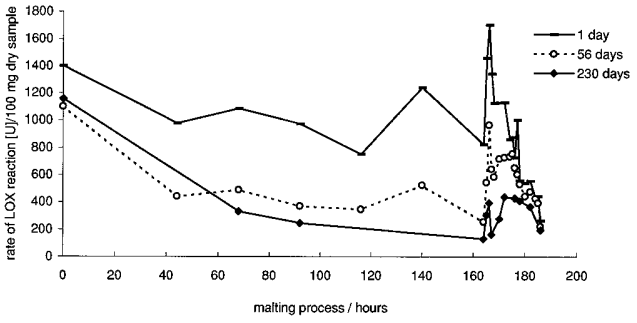

Fig. 1. Influence of storage on the rate of lipoxygenase (LOX) reaction in aqueous suspensions of barley, malt, and samples taken during malting.

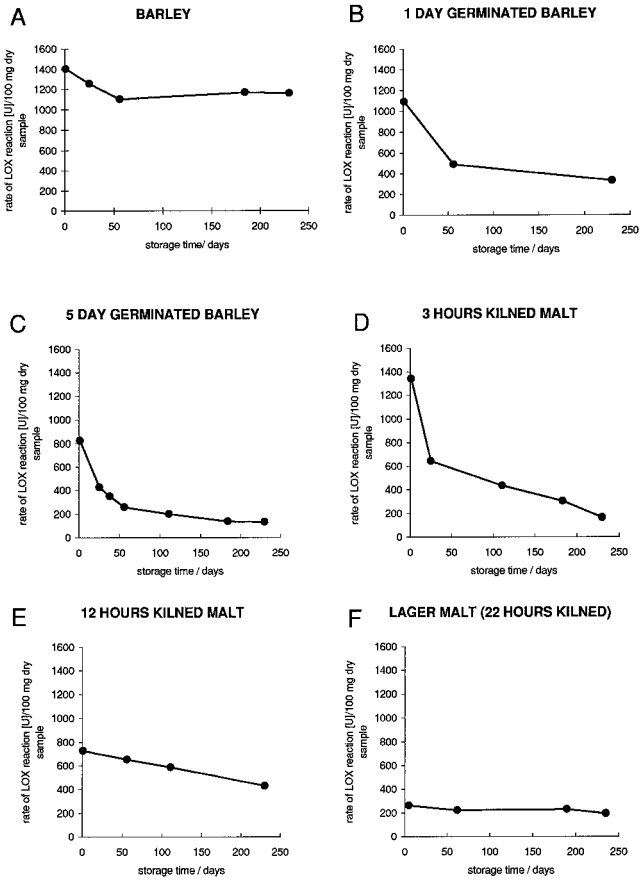

Fig. 2. Reduction in the rate of lipoxygenase (LOX) reaction in six samples during storage: barley (**A**); one day germinated sample (**B**); five day germinated samples (**C**); 3-hr kilned sample (**D**); 12-hr kilned sample (**E**); lager malt (**F**).

The results of the present study support the earlier observations on LOX where the reactions were measured several months after processing (Kaukovirta-Norja et al 1993). In light of the present data, it is evident that at such a point of storage, the rate of LOX reaction is decreased, but, as shown also in the present study, the rate of decrease differs. Thus, it may be deduced that the very strong reduction in the rate of LOX reaction during steeping and germination reported earlier for samples stored over a long period (Kaukovirta-Norja et al 1993), was a result of a faster reduction rate in these samples. However, as shown by the present data (Fig. 1), all the major trends (i.e., the reduction in the rate of LOX reaction during steeping, the invariability during germination, and the strong increase in the rate during kilning before final reduction at the end of kilning) still were similar to those of the previous study (Kaukovirta-Norja et al 1993).

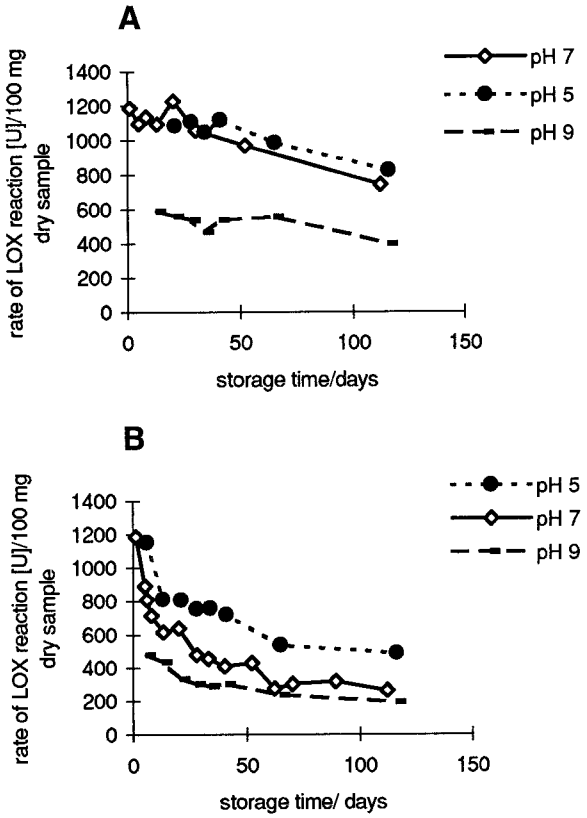

Fig. 3. Effect of storage on the rate of lipoxygenase (LOX) reaction in a malt sample kilned at a moderate temperature: stored at 5°C (**A**); stored at 23°C (**B**). Samples not freeze-dried before analysis.

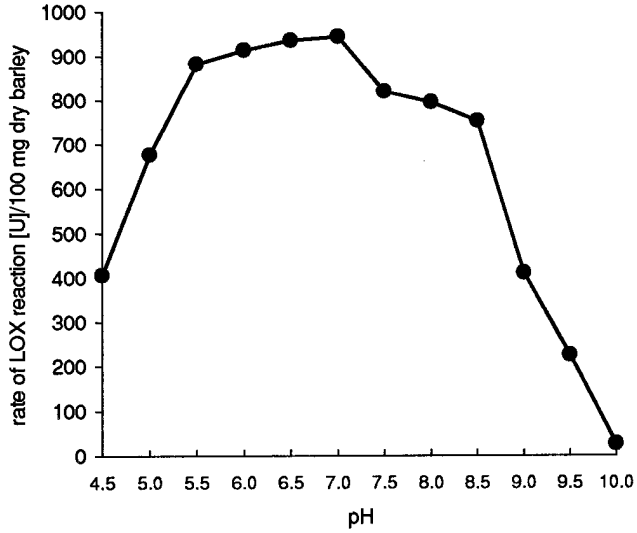

Fig. 4. Rate of lipoxygenase (LOX) reaction in aqueous barley suspension as a function of pH level.

Influence of Freeze-Drying, Storage Conditions, and Assay pH

All the samples taken during malting were freeze-dried before LOX analysis. To exclude the possible influence of freeze-drying on storage stability, a malt sample with very high rate of LOX reaction due to a moderate-temperature kilning program was analyzed. This sample was not freeze-dried before analysis. Figure 3 (a and b) shows that the effect of storage was observed also in the nonfreeze-dried sample. Furthermore, Yabuuchi and Amaha (1975) have reported that LOX-1 is resistant to freezing and freeze-drying, whereas LOX-2 is more unstable. However, the present study showed that the rate of LOX reaction was most unstable in steeping samples where LOX-1 dominated and, on the contrary, most stable in kilning samples where LOX-2 is reported to dominate. Therefore, in light of the present data, the instability of LOX reaction can not be attributed to freezing or freeze-drying.

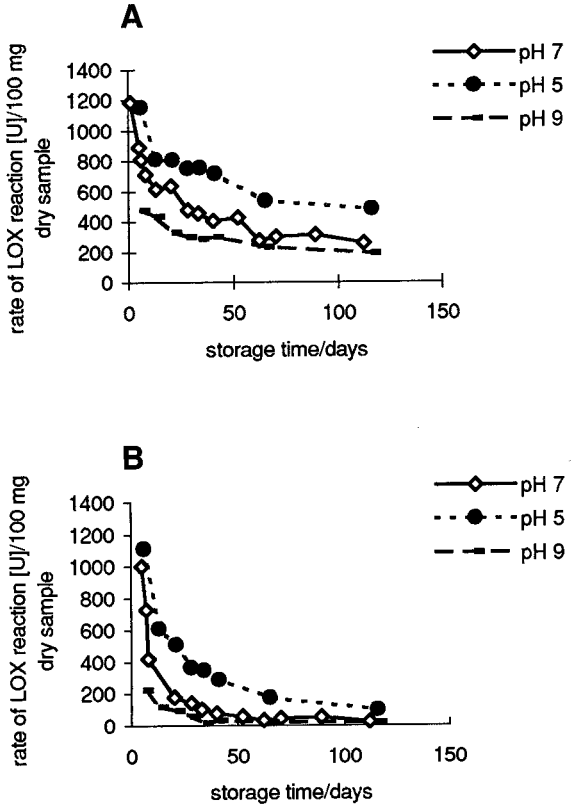

Fig. 5. Effect of storage on the rate of lipoxygenase (LOX) reaction in a sample taken during the middle of kilning: stored at 5°C (**A**); stored at 23°C (**B**).

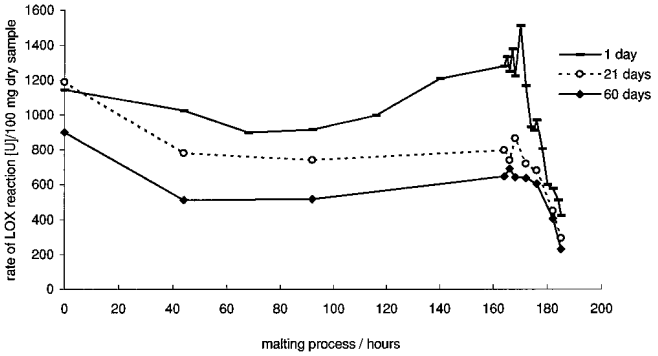

Fig. 6. Influence of storage (at 5°C) on the rate of lipoxygenase (LOX) reaction in samples first stored for six months at –20°C.

The optimum pH of purified barley LOX 1 and LOX 2 has been reported as pH 6.5 (Doderer et al 1992). Therefore, the pH range of LOX reactions in barley-water suspension was tested (Fig. 4). The highest rates were achieved at pH 7, even though activities were >90% of the maximum rate at pH 5.5–7.0. The rate of LOX reaction was clearly lower at pH <5.5 and >7.0 than at optimum pH. The pH effect was similar to that reported by Kobayashi et al (1993) during mashing where lowering the pH from 5.5 to 5.0 significantly inhibited oxidation and production of hydroperoxides. The optimum pH range for samples taken during kilning or malt seemed to be somewhat lower (5.5–6.5) and not as broad. These results are in accordance with the data of Doderer et al (1992), who found that the pH range of LOX 2, the isoenzyme dominating in malt, was narrower than the pH range of LOX 1, the iso-enzyme found in barley. Despite the differences in pH optimum, pH 7 was chosen as the standard measuring pH for all samples. The possibility that the buffering capacity of flour itself could change the assay pH was also tested, but the buffering capacity in all the buffer solutions used was observed to be strong enough. Figure 3a and b, and in addition Fig. 5a and b show that the reduction in the rate of LOX reaction with time was seen at each assay pH. In both malt and in an 11-hr kilned sample, the LOX reaction rates were initially different, but at each pH level, the reduction of activity during storage was significant. These pH measurements suggest that the reduction seen during storage in the LOX reaction would not be due to changes in the relative amounts of isoenzymes with different pH optima.

The influence of storage temperature on the LOX reaction has been reported in earlier studies (Kaukovirta-Norja et al 1995). In that data, a markedly faster reduction in rate of LOX reaction was seen in samples stored at 23°C than in those stored at 15 or 7°C. However, the present study showed that at freezing temperature (–20°C), samples were stable for six months. On the other hand, when samples were dried after freeze-storage and stored at 5°C, the reduction in the rate of LOX reaction during storage was similar to that in fresh samples (Fig. 6).

Measurement of LOX Reaction

As LA is poorly soluble in water, only a minor fraction of LA is in a monomolecular, reactive state in an aqueous environment. In highly complex cereal mixtures, LA is most probably involved in multiple interactions with saponificating and complexing agents of flour. Therefore, the effective substrate concentration in LOX assays is unknown and probably independent and far from that originally added to the assay mixture. Kernel material has been shown to form complexes with LA and prevent its use as a substrate of LOX (Lehtinen and Laakso 1997). Tightly complexed internal lipids of malt starch are more enriched in fatty acids than internal lipids of barley starch, indicating that a migration and binding of fatty acids can occur during a malting process (Kaukovirta-Norja et al 1997). While the oxygenation reaction by LOX is very seldom saturated with the LA substrate, even small changes in the ability of kernel material to bind or to complex LA can change the rate of LOX reaction. The method used resembles many real cereal processes where milling and mixing in water are involved. In such cases, not only the amount of LOX activity, but the interaction between sample and substrate, or sample and enzyme probably play a role in determining the observed rate of LOX reaction. Furthermore, even though the method used well reflects the situations in real processes, it does not give a molecular level explanation of the observed changes in LOX reactions.

DISCUSSION

Changes in the LOX reaction during malting and subsequent storage are probably caused by more than a single mechanism. Holtman et al (1996) found a decrease in LOX protein during the first day of germination, after which the amount of LOX increased during the next four days of germination due to increase in the amounts of both isoenzymes. Despite this increase in LOX protein, present results do not show a corresponding increase in LOX reaction, indicating that other mechanisms in addition to variations in the amount of LOX regulate the reaction rate in aqueous slurries. Therefore, interactions between LA substrate and the kernel material is suggested to be rate-determining in these samples. Contrary to the results presented in this article, several authors (Baxter 1982, Schwarz and Pyler 1984, Martel et al 1991, Yang et al 1993) have reported a marked increase in LOX activity during malting. However, in the above studies, LOX was a particle-free extract, which also further supports the hypothesis that a nonextractable kernel material affects the availability of the LA substrate.

Furthermore, the rapid increase in the rate of LOX reaction during the first half of kilning probably includes more than a single phenomenon. Due to the heat and air treatment, enzyme activation, enzyme synthesis or changes in kernel material that cause altered availability of substrate for enzyme, or vice versa, are all explanations that cannot be excluded. In the initial stage of kilning when temperatures are moderately high, the biological activity of the kernel increases as expressed by high production rates of CO_2 (Reinikainen et al 1996). The CO_2 production rate increases during the first 1-2 hr of kilning followed by a nearly linear decrease. Therefore, the possibility of enzyme synthesis can not be excluded. However, the available data on the changes in LOX amount is limited to the germination stage (Holtman et al 1996). Therefore this data cannot be applied to the kilning process. On the other hand, accelerated removal of water from kernel by heat and aeration is likely to influence the kernel material. Even minor physicochemical changes in kernel matrix might have significant changes in the ability of the matrix to interact with exogenous LA in aqueous slurries. During the latter part of kilning, both irreversible changes in kernel matrix and the well-known inactivation of LOX by heat (Baxter 1982, Fretzdorff and Jördens 1986, Hugues et al 1994) are probable explanations for the reduction in LOX reaction.

The changes in LOX reaction occurring during aging may also originate from changes in kernel matrix, although not necessarily from changes similar to those induced by malting. The relative stability of the barley LOX isoenzymes in extracts has been reported by Yang et al (1993). In storage, LOX-2 was more stable than LOX-1. However, in the present study, barley in which LOX-1 dominates, was the most stable sample. Furthermore, in all the samples taken during steeping or germination, the LOX reaction was similarly reduced with time even though the relations of LOX 1 and LOX 2 are known to be changed during this period (Holtman et al 1996). This suggests that the observed changes in LOX reaction during storage of samples is more likely due to changes in kernel material than in activities of isoenzymes. Structural and organizational changes within the matrix may change the kernel's ability to stabilize LOX itself, or its ability to protect LA from LOX attack when the kernel is ground and suspended in aqueous slurries. Enzymes (e.g., amylases and proteases) that are known to remain stable within similar storage times have substrates with more hydrophilic character. This also supports the idea that with poorly soluble substrates, the observed reaction rates depend on the physical state of the substrate, that is, its effective concentration.

CONCLUSIONS

The rate of LOX reaction in malt is mainly affected by two factors: the malting process and the subsequent time of storage. The most critical stage of malting in determining LOX reaction was kilning. During the first two to three weeks of storage, the rate of LOX reaction reduced to a more stable level. The effect of storage seemed not to be dependent on malting program.

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