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## Characterization of 9-Fatty Acid Hydroperoxide Lyase-Like Activity in Germinating Barley Seeds That Transforms 9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic Acid into 2(*E*)-Nonenal

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Previously, we reported that 2(*E*)-nonenal, having a low flavor threshold (0.1 ppb) and known as the major contributor to a cardboard flavor (stale flavor) in stored beer, is produced by lipoxygenase-1 and a newly found factor named 9-fatty acid hydroperoxide lyase-like (9-HPL-like) activity in malt. To assess the involvement of 9-HPL-like activity in beer staling, we compared the values of the wort nonenal potential, an index for predicting the staleness of beer, with the lipoxygenase and 9-HPL-like activity of 20 commercial malts. There was a significant correlation between the malt 9-HPL-like activity and the values of wort nonenal potential ( $r = 0.53$ ,  $P < 0.05$ ), while the correlation between malt lipoxygenase activity and the wort nonenal potential was statistically insignificant. Analysis of the partially purified 9-HPL-like activity from embryos of germinating barley seeds indicated that 9-HPL-like activity consisted of fatty acid hydroperoxide lyase and 3*Z*:2*E* isomerase.

**Key words:** fatty acid hydroperoxide lyase; barley; 2(*E*)-nonenal; 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 3*Z*:2*E* isomerase

Flavor-active compounds derived from oxygenated lipids have a large impact on the quality of foods and beverages.<sup>1)</sup> Malt contains lipids and lipid-oxidizing enzymes, which in turn produce flavor-active oxygenated species during mashing. Among them, 2(*E*)-nonenal has a very low flavor threshold in beer (0.7 nM, 0.1 ppb),<sup>2)</sup> and is considered to be the major contributor to the “cardboard flavor” that arises when a beer is stored at high temperature or for a prolonged period. There is correlation among malt lipoxygenase (LOX), the wort nonenal potential, 2(*E*)-nonenal that develops in beer after storage, and the sensory scores of staleness characterizing the beer as having “cardboard flavor”.<sup>3)</sup> The test of wort nonenal potential, widely used by brewers and brewing chemists to estimate the quality of malt related to beer flavor stability, is a kind of

forcing test to measure the potential of wort to form 2(*E*)-nonenal. The values are expressed as the concentration (ppb) of 2(*E*)-nonenal in the heat-treated wort samples. Very successful malt selection and malting methods for improving the flavor stability of beer by reducing the values of the wort nonenal potential have been developed.<sup>4,5)</sup> Although we often use 2(*E*)-nonenal as a marker for beer staling, the biochemical background of the formation of 2(*E*)-nonenal during mashing is not fully understood. Therefore, in a previous study, we characterized the factors involved in the production of 2(*E*)-nonenal during mashing and found that 2(*E*)-nonenal is produced by the cascade reaction of barley lipoxygenase-1 (LOX-1) and malt 9-fatty acid hydroperoxide lyase-like activity (9-HPL-like activity) in malt.<sup>6)</sup> 9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPOD) produced by LOX-1 was further cleaved by 9-HPL-like activity to generate 2(*E*)-nonenal during mashing. 9-HPL-like activity was like that of an enzyme because it was sensitive to proteinase-K treatment, and was moreover completely terminated when boiled. Malt also contained heat-labile 13-fatty acid hydroperoxide lyase-like activity (13-HPL-like activity) that transformed 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD) into hexanal. The 9- and 13-HPL-like activities were considered to be distinct factors, because they had different sensitivity to proteinase-K.

Fatty acid hydroperoxide lyase (HPL), ubiquitously found in the plant kingdom, catalyzes the cleavage reaction of fatty acid hydroperoxides into oxo-acids and aldehydes. Intensive studies on HPL in dicots have revealed that HPL is a member of the cytochrome P450 family, which plays an important role in the interaction among plant-herbivores by producing phytooxylipins.<sup>7,8)</sup> HPL is grouped by substrate specificity into two groups, 13-HPL (CYP74B) and 9-/13-HPL (CYP74C).<sup>9,10)</sup> 9-/13-HPL cleaves 9-HPOD, 9(*S*)-hydroperoxy-10(*E*), 12(*Z*), 15(*Z*)-octadecatrienoic acid, 13-HPOD, and 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid

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**Abbreviations:** HPL, fatty acid hydroperoxide lyase; 9-HPOD, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; LOX, lipoxygenase

into 3(*Z*)-nonenal, 3(*Z*),6(*Z*)-nonadienal, hexanal, and 3(*Z*)-hexenal respectively. On the other hand, 13-HPL preferentially cleaves 13-HPOD and 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid into hexanal and 3(*Z*)-hexenal respectively. 3(*Z*)-Nonenal, 3(*Z*),6(*Z*)-nonadienal, and 3(*Z*)-hexenal are spontaneously or enzymatically isomerized into 2(*E*)-nonenal, 2(*E*),6(*Z*)-nonadienal, and 2(*E*)-hexenal respectively. HPL affects the flavor characteristics of food products by changing the constituents of volatile aldehydes.<sup>11–13</sup> Although there are several studies on the effect of dicot HPL on food quality, in monocots, especially in cereal products including malt, there have been few studies on the presence of HPL activity, and none on its role in the production of volatile aldehydes in foods until our report.<sup>6</sup> The only published report to date is on the presence of the cDNA clone that encodes for 13-HPL in barley. The recombinant protein of this cDNA expressed in *E. coli* was shown to cleave only 13-HPOD and 13-HPOT into hexanal and hexenal, but had no activity against 9-HPOD or 9-HPOT,<sup>14</sup> so that information on the fatty acid hydroperoxide lyase that catalyzes the transformation of 9-HPOD and 9-HPOT in monocots is still missing. The 9-HPL-like activity found in malt is considered to be possibly the first HPL that cleaves 9-HPOD in monocots.

In this study, we first analyzed the correlation between 9-HPL-like activity in malt and the values of the wort nonenal potential. Secondly, we partially purified and characterized the biochemical properties of 9-HPL-like activity from embryos of germinating barley seeds. There was significant correlation between 9-HPL-like activity and the wort nonenal potential, which implies the involvement of 9-HPL-like activity in the formation of cardboard flavor. Biochemical analysis revealed that 9-HPL-like activity consisted of HPL and 3*Z*:2*E* isomerase. Our study indicates the presence of HPL that cleaves 9-HPOD in monocots and its importance for the qualities of foods and beverages made from cereals.

## Materials and Methods

**Chemicals.** Nordihydroguaiaretic acid was from ICN Biomedicals (Ohio, U.S.A.). Salicylic acid, 2(*E*)-nonenal, and hexanal were from Wako Pure Chemical Industries (Osaka, Japan). 3(*Z*)-Nonenal was from Larodan Fine Chemicals (Malmö, Sweden). 9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPOD) was produced through the reaction of recombinant barley LOX-1 (rLOX-1)<sup>15</sup> and linoleic acid as follows: rLOX-1 (22  $\mu$ kat) was diluted with 300 ml of 0.1 M sodium acetate buffer (pH 6.0) and incubated with 15 ml of 40 mM of linoleic acid dissolved in ethanol at 20 °C for 5 min. The reaction was terminated by adding 30 ml of CH<sub>2</sub>Cl<sub>2</sub> and 25 ml of 5 M NaCl, and the mixture was vigorously shaken. After standing for 10 min, the CH<sub>2</sub>Cl<sub>2</sub> layer was recovered and dried under nitrogen.

The sample was dissolved in 10 ml of hexane/ether (9:1) and applied to a silica-gel column (silica-gel 60, Merk; particle size, 0.063–0.2 mm; column size, 1 cm in diameter  $\times$  5 cm long) that was pre-equilibrated with hexane/ether (9:1). After the column was washed with 60 ml of hexane/ether (9:1), 9-HPOD was eluted with hexane/ether (1:1) and fractionated. The fractions were analyzed with TLC and then visualized with both UV and iodide. Fractions containing 9-HPOD free from linoleic acid were collected, and the solvent was dried under nitrogen. 9-HPOD was dissolved in ethanol at a concentration of 20 mM and stored in aliquots at –135 °C until use. The purity of 9-HPOD was determined using straight-phase HPLC as described previously.<sup>6</sup>

**Enzyme assays of 9-HPL-like activity and 3*Z*:2*E* isomerase.** 9-HPL-like activity was measured as follows. The enzyme solution was diluted with 0.1 M Tris–HCl (pH 7.0) to 100  $\mu$ l in an eppendorf tube, and 5  $\mu$ l of 2 mM 9-HPOD dissolved in ethanol was added. The reaction mixture was incubated at 30 °C for 5 min and the reaction was stopped by the addition of ice-cold ethanol containing 40 ppb 1-nonanol (0.9 ml). After chilling on ice for 5 min, it was centrifuged at 10,000  $\times$  *g* at 4 °C for 1 min. The supernatant was transferred to a vial containing 6.5 ml of distilled water and 3 g of sodium chloride and sealed with a silicon-coated cap. 2(*E*)-Nonenal was trapped by inserting a SPME fiber (100  $\mu$ m membrane-thick polydimethylsiloxane solid phase micro extraction fiber probe, Spelco, PA, U.S.A.) into the headspace of the vial at 40 °C for 15 min. The probe was then introduced into the injection port and thermally desorbed at 260 °C for 5 min onto a DB-1 column (liquid phase = dimethylpolysiloxane, 30 m  $\times$  0.25 mm, film thickness = 1  $\mu$ m, J&W Scientific, CA, U.S.A.). GC–MS analysis was conducted with the Hewlett Packard HP6890/MSD system. The selected ions used were *m/z* = 69 and 70. The flow rate of helium gas was 1.0 ml/min. The temperature of the oven was maintained at 60 °C for 1 min, and then raised by 5 °C/min to 260 °C. Calibration curves were derived from the authentic 2(*E*)-nonenal solution containing the internal standard (1-nonanol). The concentration of 2(*E*)-nonenal in each sample was measured by the peak area relative to that of the internal standard. 9-HPL-like activity was expressed as the rate of formation of 2(*E*)-nonenal (in katal).

3*Z*:2*E* Isomerase activity was measured as follows. The enzyme solution was diluted with 0.1 M Tris–HCl (pH 7.0) to 100  $\mu$ l in an eppendorf tube and 5  $\mu$ l of 2 mM 3(*Z*)-nonenal (ethanol solution) was added. After the reaction mixture was incubated at 30 °C for 20 min, products were extracted with 200  $\mu$ l of ether containing 50 ppb 1-nonanol as an internal standard. A portion of ether layer (2  $\mu$ l) was subjected directly onto GC equipped with the same column as previously described. GC–MS analysis was conducted similarly, except for the

temperature program (120 °C for 1 min, then raised by 5 °C/min to 150 °C, and finally 40 °C/min to 300 °C). The amount of 2(*E*)-nonenal was measured as described. 3*Z*:2*E* isomerase activity was expressed as a rate of the formation of 2(*E*)-nonenal (in katal).

*Analysis of 9-HPL-like activity, lipoxygenase and nonenal potential of 20 commercial malts.* A total of 20 commercial malts from domestic, European, and Australian varieties were used for the analysis. Malt enzyme extract was prepared as previously reported.<sup>6)</sup> Briefly, malt meal (1 g) was stirred with 10 ml of 0.1 M potassium phosphate buffer (pH 6.0), 0.15% TritonX-100 for 1 h at room temperature. After centrifuging at 10,000 × *g* for 20 min, the supernatant was used as an enzyme extract. 9-HPL-like activity was measured as described. LOX activity was measured spectrophotometrically as previously reported.<sup>6)</sup> LOX activity (in katal) was expressed as the rate of formation of conjugated diene chromophore ( $\epsilon = 25,000$ ). Wort was prepared using the method of the European Brewery Convention,<sup>16)</sup> using a Löchner LB8 mashing machine (Löchner, Germany). The wort nonenal potential was determined using Drost's method.<sup>3)</sup> Statistical analysis was carried out with a SPSS 9.0J for Windows software (SPSS Japan, Tokyo, Japan).

*Partial purification of 9-HPL-like activity.* One gram of embryos was excised from germinating barley seeds (*Hordeum vulgare*, cv. Haruna nijo), and homogenated in 10 ml of ice-cold 0.1 M sodium acetate, 5 mM EDTA, 0.1% Tween20, 5 mM DTT, and EDTA-free Complete protease inhibitor™ (Roche, Basel, Switzerland), pH 5.5. After centrifuging at 10,000 × *g* at 4 °C for 1 h, the supernatant was desalted with a Hitrap desalting column (Amersham Pharmacia Biotech) into 10 mM Tris-HCl (pH 7.2), 0.1% Tween 20, and 1 mM  $\beta$ -mercaptoethanol. An eluent was applied to a Resource Q column (Amersham Pharmacia Biotech; diameter = 6.4 mm, length = 30 mm, bed volume = 1 ml) equilibrated with 10 mM Tris-HCl (pH 7.2), 0.1% Tween20, and 1 mM  $\beta$ -mercaptoethanol, and eluted by a NaCl gradient (0–1 M), using an AKTA purifier FPLC system (Amersham Pharmacia Biotech) at 4 °C. Fractions of 0.5 ml were collected and assayed for 9-HPL-like activity. 9-HPL-like activity was also purified with Mono-P 5/50 GL chromatofocusing chromatography (Amersham Pharmacia Biotech), Resource S cation-exchange chromatography (1 ml) (Amersham Pharmacia Biotech), or Hydroxyapatite Bio-Gel HT Gel chromatography (Bio-Rad Laboratories, U.S.A.).

*Characterization of 9-HPL-like activity.* GC-MS analysis of aldehydes produced by 9-HPL-like activity was as follows: Partially purified 9-HPL-like activity (30  $\mu$ g) was diluted to 1 ml of 0.1 M Tris-HCl (pH 7.0) in a GC vial and mixed with 9-HPOD at a final concentration of 0.1 mM. After capping, SPME fiber was

inserted either immediately, or after incubation at 30 °C for 5 min. Aldehydes accumulated in the vial were trapped with SPME fiber at 30 °C for 5 min and subjected to GC-MS. The condition of GC oven was same as that for the 3*Z*:2*E* isomerase assay. Products were identified by mass-spectrum analysis and the coincidence of retention time with authentic compounds.

To analyze the substrate effect on velocity, 9-HPL-like activity was assayed at final concentrations of 0.008–0.84 mM with concentrated (×20) 9-HPOD dissolved in ethanol. The apparent  $K_m$  value was estimated by Lineweaver-Burk reciprocal plots. To test the effect of salicylic and nordihydroguaiaretic acids on 9-HPL-like activity, concentrated solutions (×20) were added to the reaction mixture at a final concentration of 0.1 mM or 1 mM. To analyze the effect of pH, 0.1 M potassium phosphate buffer (pH 5.0–8.0), 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH (pH 5.5–6.5), and 0.1 M Tris-HCl (pH 8.0) were used instead of 0.1 M Tris-HCl (pH 7.0).

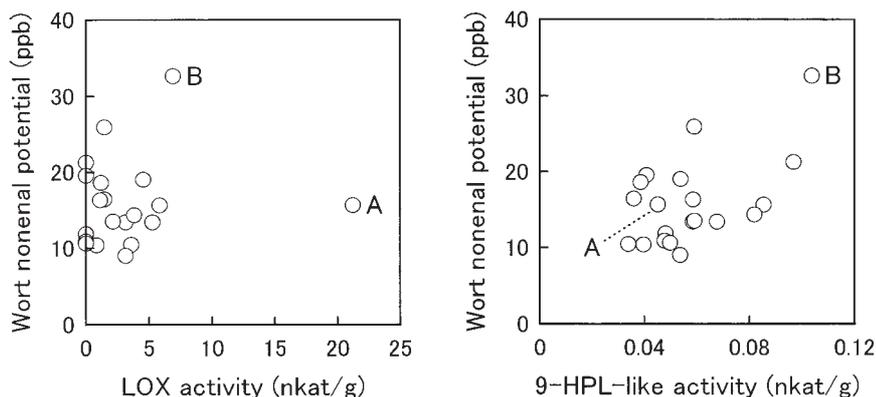
The molecular weight of 9-HPL-like activity was estimated by gel-chromatography. A portion of partially purified 9-HPL-like activity (4 pkat) was run on a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech; bed diameter = 10 mm, length = 300 mm, bed volume = 24 ml) using an AKTA purifier FPLC system (Amersham Pharmacia Biotech) at 20 °C, and fractions of 0.5 ml were collected and assayed for 9-HPL-like activity. The marker proteins (all from Amersham Pharmacia Biotech) used were ovalbumin (hen's egg, 43 kDa), albumin (bovine serum, 67 kDa), aldolase (rabbit muscle, 158 kDa), and catalase (bovine liver, 232 kDa). The elution profiles were analyzed with Unicorn software, Version 4 (Amersham Pharmacia Biotech).

Substrate specificity was determined spectrophotometrically by monitoring a decrease in the substrates as follows: 9-HPOD or 13-HPOD was diluted to 1 ml of 0.1 M Tris-HCl (pH 7.0) in a cell at a final concentration of 40  $\mu$ M. An aliquot of the partial purified fraction was mixed, and absorbance at 234 nm was monitored at room temperature.

## Results

### *Analysis of correlation between wort nonenal potential (beer staling index), 9-HPL-like activity, and the LOX activity of malt*

To examine the relation between 9-HPL-like activity and malt quality with regard to beer staling, the enzymatic activities of 9-HPL-like activity, malt LOX activity, and the values of the wort nonenal potential of 20 randomly sampled commercial malts were compared. We observed the production of 2(*E*)-nonenal but not 3(*Z*)-nonenal when malt extracts were incubated with 9-HPOD. The wort nonenal potential, an index widely used by brewers and brewing chemists to evaluate the quality of malt related to beer staling, is a kind of



**Fig. 1.** Correlation between the Wort Nonenal Potential (Beer Staling Index), 9-HPL-Like Activity, and LOX Activity of 20 Commercial Malts. The wort nonenal potential, 9-HPL-like activity, and LOX activity were measured for 20 commercial malts. The correlation between the malt 9-HPL-like activity and the values of wort nonenal potential was statistically significant ( $r = 0.53$ ,  $P < 0.05$  by Pearson's correlation coefficient). Malt A, with half the wort nonenal potential of Malt B, had three times more LOX activity, while Malt A had 2.5 times less 9-HPL-like activity. Symbols: A, Malt A; B, Malt B.

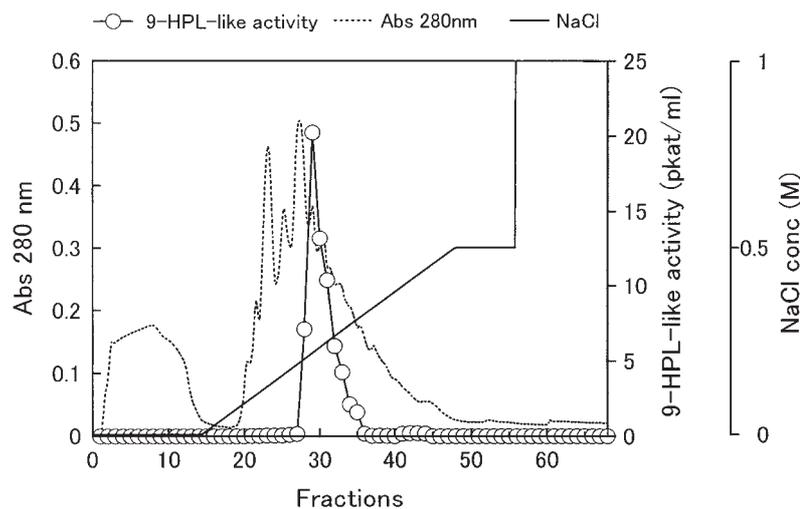
forcing test to measure the potential of wort to form 2(*E*)-nonenal; the values are expressed as the concentration of 2(*E*)-nonenal in heat-treated wort samples.<sup>3)</sup> There is abundant evidence that the values of the wort nonenal potential correlate positively with the formation of 2(*E*)-nonenal after the beer storage, sensory scores of staleness of beer, and how much a beer is described as having a "cardboard flavor".<sup>4,5)</sup> As clearly shown in Fig. 1, there was a significant correlation between the malt 9-HPL-like activity and the values of the wort nonenal potential ( $r = 0.53$ ,  $P < 0.05$  by Pearson's correlation coefficient). In contrast, the correlation between malt LOX activity and the wort nonenal potential was statistically insignificant. However, since there are two isozymes (LOX-1 and LOX-2) in germinating barley seeds,<sup>15)</sup> and in the present study we measured only the total LOX activity, we might observe significant correlation between malt LOX-1 and the wort nonenal potential if we specifically measure malt LOX-1 activity. It is interesting that "Malt A" with half the wort nonenal potential of "Malt B" had three times more LOX activity. On the other hand, "Malt A" had 2.5 less 9-HPL-like activity than "Malt B". This implies that although the levels of 9-HPL-like activity (0.033–0.103 nkat/g) were much lower than those of LOX activity (detection limit to 21 nkat/g) in malt, 9-HPL-like activity might be the determining factor of the values of wort nonenal potential.

#### *Partial purification and characterization of 9-HPL-like activity*

Next we attempted to purify and characterize 9-HPL-like activity. We did not use malt for enzyme purification, because kilning (the heat-drying process for germinating barley) causes modifications of malt proteins, which disturb the structure analysis of the target protein at the molecular level, such as N-terminal amino acid sequence or LC-MS analysis. Instead, we used embryos of germinating barley. A preliminary experi-

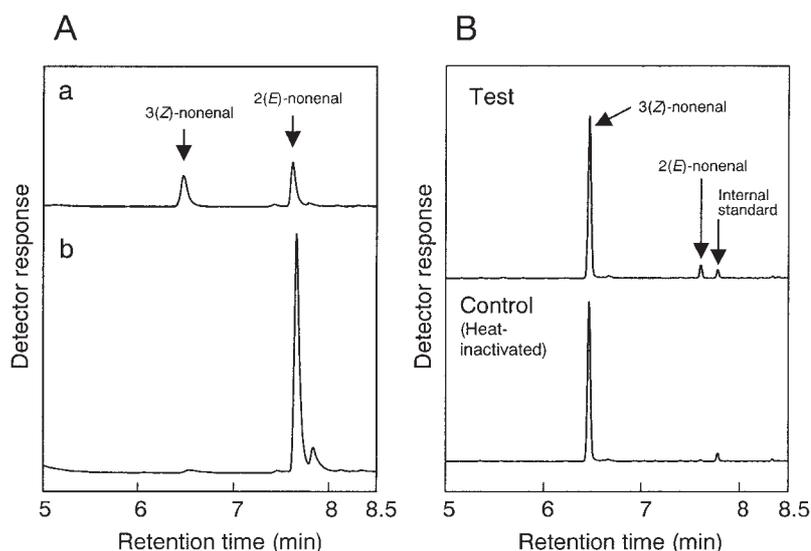
ment showed that 9-HPL-like activity was localized only in embryos, and not elsewhere in the germinating barley seeds. Differently from the enzyme extract of malt, dilution of an extract from embryos resulted in an increase in the specific activity of 9-HPL-like activity; afterwards, we confirmed that this was due to the presence of inhibitory factors. This effect was observed for both the crude extract and the fraction obtained by gel-filtration ( $M_r > 5,000$ ). Partial purification by Resource Q anion-exchange column chromatography led to a single active-peak fraction of 9-HPL-like activity that was free from inhibitory factors (Fig. 2). The dilution effect was observed no more in this sample. We confirmed that the production of 2(*E*)-nonenal increases linearly with increases in enzyme extract. In no fraction was production of 3(*Z*)-nonenal observed.

This partially purified fraction contained both HPL and 3*Z*:2*E* isomerase. Figure 3A shows the GC-MS analysis (total ion chromatograms) of the products formed by incubation of 9-HPOD with a partially purified fraction. Immediately after incubation, we observed the production of both 3(*Z*)-nonenal ( $m/z = 122, 111, 96, 84, 69, 55, 41$ ) and 2(*E*)-nonenal ( $m/z = 122, 111, 96, 83, 70, 55, 41$ ), while after the reaction was continued for 5 min, 3(*Z*)-nonenal disappeared and the major product observed was 2(*E*)-nonenal. We found neither 3(*Z*)-nonenal nor 2(*E*)-nonenal in the reaction product from the boiled fraction with 9-HPOD. This result indicates that the partially purified sample contains both HPL and isomerization factor. The isomerization factor was an enzyme, because the partially purified fraction transformed 3(*Z*)-nonenal into 2(*E*)-nonenal, but it was inactivated by boiling (Fig. 3B). Table 1 summarizes the results of partial purification of 9-HPL-like activity. Following Resource Q anion-exchange column chromatography, the specific activity of 9-HPL-like activity increased 987-fold, and the recovery rate was 1,025%. But these figures are overestimated due to the presence of the inhibitory



**Fig. 2.** Partial Purification of 9-HPL-Like Activity from the Embryos of Germinating Barley Seeds by Anion Exchange Chromatography.

Desalted enzyme extract was applied to a Resource Q column equilibrated with 10 mM Tris-HCl (pH 7.2), 0.1% Tween20, and 1 mM  $\beta$ -mercaptoethanol, and eluted by a NaCl gradient (0–1 M). Fractions of 0.5 ml were collected and assayed for 9-HPL-like activity. Symbols: open circles, 9-HPL-like activity; solid line, concentration of NaCl (M); dotted line, absorbance at 280 nm.

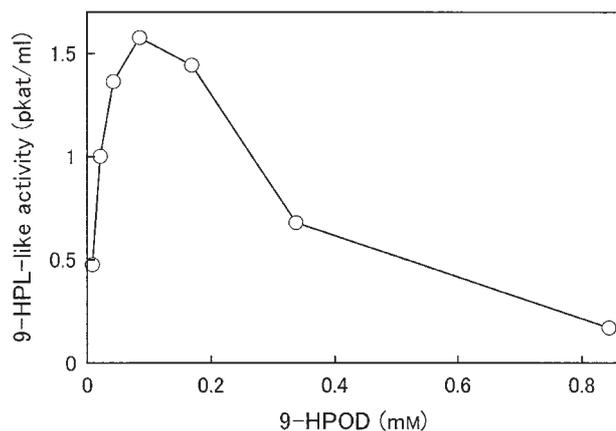


**Fig. 3.** GC-MS Analysis of 9-HPL-Like Activity and 3Z:2E Isomerase.

A, Partially purified 9-HPL-like activity was mixed with 9-HPOD at a final concentration of 0.1 mM in a GC vial. After capping, a SPME fiber was inserted either immediately (a), or after incubating at 30 °C for 5 min (b), into the headspace of the vial. Aldehydes accumulated in a GC vial were trapped with a SPME fiber for 5 min, and subjected to GC-MS analysis. Total ion chromatograms are presented. B, Partially purified 9-HPL-like activity (test and boiled control) was incubated with 3(Z)-nonenal at 30 °C for 20 min, and the products were extracted with ether containing an internal standard (1-nonanol). A portion of ether layer was subjected to GC-MS analysis (SIM mode; select ion,  $m/z = 69$  and 70).

**Table 1.** Partial Purification of 9-HPL-Like Activity

Fractions	Total protein (mg)	9-HPL-like activity		3Z:2E Isomerase activity	
		Specific activity (pkat/mg)	Recovery (%)	Specific activity (pkat/mg)	Recovery (%)
Crude extract	26	0.3	100	11	100
Gel filtration	24	0.63	194	11	92
Resource Q peak fraction	0.27	296	1025	180	18



**Fig. 4.** Substrate-Velocity Plot of Partially Purified 9-HPL-Like Activity.

The velocity of 9-HPL-like activity was measured at a concentration of 0.0084–0.84 mM. The apparent  $K_m$  value was estimated to be 23  $\mu$ M.

factors. 3Z:2E Isomerase was co-purified with 9-HPL-like activity. The specific activity was increased to 16-fold, and the recovery rate was 18%. To characterize HPL in 9-HPL-like activity, we further attempted several purification methods to separate HPL and 3Z:2E isomerase, but these activities could not be separated by chromatofocusing, cation-exchange, or hydroxyapatite chromatography.

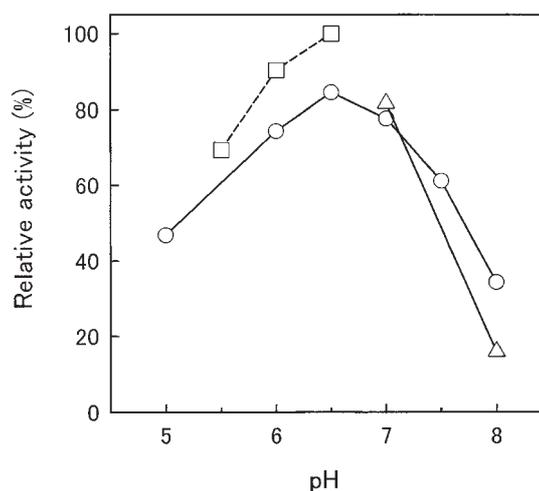
Using this partially purified fraction which contained both HPL and 3Z:2E isomerase activity, we performed several biochemical analyses to characterize 9-HPL-like activity. In every characterization, we monitored the production of both 3(Z)-nonenal and 2(E)-nonenal, but we observed only the production of 2(E)-nonenal. First we examined the substrate-velocity plot (0.008–0.84 mM) of 9-HPL-like activity (Fig. 4). The velocity of 9-HPL-like activity increased with a rise in substrate concentration from 0 to 84  $\mu$ M, while it decreased more than 170  $\mu$ M. At a concentration of 0.85 mM, activity decreased to 11% of maximum, showing that 9-HPOD acts as a suicide substrate. The apparent  $K_m$  value for 9-HPL-like activity was estimated to be 23  $\mu$ M, similar to that of HPL in dicots. The apparent  $K_m$  values for 13-HPOD were 14.6  $\mu$ M with tea leaf HPL,<sup>17)</sup> and 20.8 and 22.1  $\mu$ M with HPL I and II of bell pepper fruits.<sup>18)</sup>

Next, gel filtration chromatography was performed on a portion of partially purified 9-HPL-like activity to estimate its molecular weight (data not shown). Again, we could not separate HPL and 3Z:2E isomerase. The position of the peak active fraction coincided with the eluting point of aldolase, showing that the molecular weight of 9-HPL-like activity is approximately 160 kDa. The molecular weight of bell pepper fruit fatty acid hydroperoxide lyase was 170 kDa, consisting of a trimer of 55 kDa polypeptide,<sup>18)</sup> and that of soybean HPL was 240–260 kDa.<sup>19)</sup>

Next, we examined the effect of reagents and pH on 9-HPL-like activity. Nordihydroguaiaretic acid reduced

**Table 2.** Effect of Reagents on 9-HPL-Like Activity

Reagent	Concentration	Relative activity (%)
None		100
Salicylic acid	0.1 mM	84
	1 mM	6.4
Nordihydroguaiaretic acid	0.1 mM	70
	1 mM	0
KCl	1 M	62



**Fig. 5.** Effect of pH on 9-HPL-Like Activity.

9-HPL-like activity was measured in either 0.1 M potassium phosphate buffer (open circle, pH 5.0–8.0), 0.1 M MES-KOH (open square, pH 5.5–6.5), or 0.1 M Tris-HCl (open triangle, pH 7.0–8.0).

9-HPL-like activity to 70% at a concentration of 0.1 mM, and completely inhibited it at a concentration of 1 mM. Salicylic acid reduced the activity to 84% and 6.4% at concentrations of 0.1 mM and 1 mM respectively. Increasing the salt concentration (1 M KCl) resulted in inhibition of 9-HPL-like activity to 62% (Table 2). The pH-velocity plot of 9-HPL-like activity showed a typical bellshape (pH 5–8), with maximum activity at pH 6.5 in the potassium phosphate buffer (Fig. 5). Finally, we examined the substrate specificity of partially purified 9-HPL-like activity by monitoring the decomposition of hydroperoxide substrates. The relative activity toward 9-HPOD and 13-HPOD was 100:34.

## Discussion

This paper characterizes 9-HPL-like activity in relation to the flavor stability of beer, and shows the biochemical properties of partially purified 9-HPL-like activity from embryos of germinating barley seeds. We found a positive correlation between 9-HPL-like activity and the value of wort nonenal potential, an index for predicting the staleness of stored beer. The staleness of beer caused by lipid oxidation during mashing is often described as a “cardboard flavor”, and 2(E)-nonenal is considered to be the major contributor of this flavor.<sup>3–5)</sup>

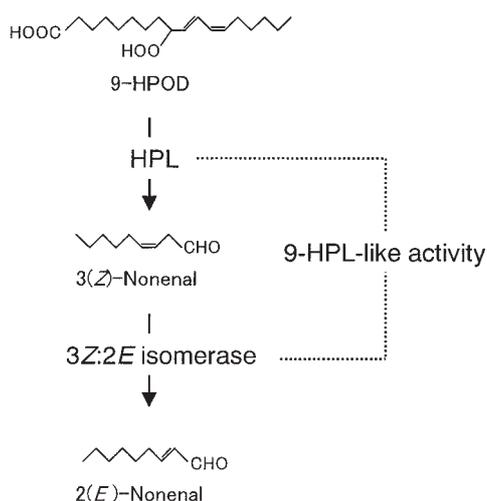
Recently, we discovered barley germplasms that lack LOX-1, and the following brewing trial with malt made from a malting barley crossed with one of these germplasms revealed LOX-1 to have a significant effect on the flavor stability of beer.<sup>20</sup> Beer brewed with LOX-less malt showed a higher sensory score with fewer “cardboard flavor” comments after storage compared to the control line. Because LOX-1 produces only 9-HPOD, we confirmed that the flavor compounds derived from the decomposition or transformation of 9-HPOD were the source of the cardboard flavor. The fact that 9-HPL-like activity cleaves 9-HPOD to produce 2(*E*)-nonenal, and the fact that activity positively correlates with the wort nonenal potential imply that not only LOX-1, but also 9-HPL-like activity might be involved in the formation of cardboard flavor.

Next, the possible involvement of 9-HPL-like activity in beer flavor stability created the necessity of biochemical and molecular analyses of this factor. Hence, we attempted purification of this factor from the embryos of germinating barley seeds. From the biochemical analysis of partially purified 9-HPL-like activity, it was shown that the activity consisted of HPL and 3*Z*:2*E* isomerase (Fig. 6). Further attempts to separate these activities failed, and hence we performed several biochemical analyses using partially purified 9-HPL-like activity which contained both HPL and 3*Z*:2*E* isomerase. From these analyses, we obtained further evidence that 9-HPL-like activity contained HPL. First, 9-HPL-like activity was inhibited by nordihydroguaiaretic and salicylic acids (Table 2). Because 3(*Z*)-nonenal was not observed in all tests, it is thought that activity was inhibited at the catalytic step of HPL. Lipophilic antioxidants such as nordihydroguaiaretic acid are

thought to prevent the radical-formation step of the catalytic activity of HPL,<sup>17</sup> and salicylic acid is thought to act as a metal-chelating reagent.<sup>21</sup> Secondly, 9-HPL-like activity was inhibited by a higher substrate concentration (Fig. 4). HPL is thought to convert fatty acid hydroperoxide into radical species, which in turn destroy their own SH group, essential to catalytic activity, thus inhibiting catalytic activity irreversibly.<sup>17</sup>

At present, we do not have any information on HPL in 9-HPL-like activity as 9-/13-HPL. But, the presence of HPL which cleaves 9-HPOD in germinating barley seeds reminds one of the existence of both 13-HPL (CYP74B) and 9-/13-HPL (CYP74C) in barley. The HPL in 9-HPL-like activity characterized in this study differed from barley 13-HPL<sup>14</sup> on several points. First, recombinant enzyme of barley 13-HPL expressed in *E. coli* did not cleave 9-HPOD. Secondly, the pH-velocity plots of 9-HPL-like activity and the barley recombinant 13-HPL were different. Barley 13-HPL had slightly higher activity at pH 8.0 than at pH 7.0 in a 0.1 M potassium phosphate buffer, whereas the level of 9-HPL-like activity measured at pH 8.0 in the same buffer was 44% of that measured at pH 7.0 (Fig. 5). Thirdly, 9-HPL-like activity was inhibited by a higher salt concentration, while barley 13-HPL was enhanced (Table 2). In a previous report, we reported the presence of 13-HPL-like activity that cleaved 13-HPOD into hexanal, in addition to 9-HPL-like activity. These activities were considered to be distinct because they showed different sensitivity to proteinase-K.<sup>6</sup> The production of 2(*E*)-nonenal was reduced by proteinase-K treatment, while hexanal production was not affected by the same treatment. Assuming that 9-HPL-like activity is 9-/13-HPL, part of the production of hexanal would be reduced by proteinase-K treatment; however, this effect would not be observed because the activity of 13-HPL-like activity was 100 times higher than that of 9-HPL-like activity. In our preliminary experiment, we attempted to separate 9- and 13-HPL-like activities by Resource Q anion exchange chromatography, which directly indicates that these two activities are distinct factors. However, we did not find a peak of 13-HPL-like activity, presumably for the presence of other enzymes which consume 13-HPOD. Whether 9- and 13-HPL-like activities correspond to 9-/13-HPL and 13-HPL is to be the subject of further work.

Although there are several reports showing the relation between the quality of foods and the flavor compounds derived from 13-HPOD or 13-HPOT by the enzymatic action of 13-HPL or 9-/13-HPL,<sup>11–13</sup> the effect of flavors developed from 9-HPOD by the action of 9-/13-HPL on food quality is less fully characterized.<sup>6,22</sup> This does not mean that the off-flavors derived from 9-HPOD are less important, because as described, 2(*E*)-nonenal is thought to have a large influence on the formation of the stale flavor of beer during storage. Additionally, in rice, 2(*E*)-nonenal has been reported to be a decisive element in determining the off-flavor of



**Fig. 6.** Pathway for the Production of 2(*E*)-Nonenal from 9-HPOD with 9-HPL-Like Activity.

9-HPOD produced from the reaction of LOX-1 and linoleic acid was cleaved into 3(*Z*)-nonenal with HPL. 3(*Z*)-Nonenal was further isomerized into 2(*E*)-nonenal with 3*Z*:2*E* isomerase. 9-HPL-like activity consisted of HPL and 3*Z*:2*E* isomerase.

milled rice.<sup>23)</sup> It is intriguing that LOX3, the major LOX found in the bran of rice seeds, specifically produces 9-HPOD,<sup>24)</sup> as with barley LOX-1 in seeds or malts that produce only 9-HPOD.<sup>15)</sup> We speculate that in cereals, including barley and rice, the 9-LOX and 9-/13-HPL cascade pathway that generates 2(*E*)-nonenal from linoleic acid might have a significant effect on deterioration in flavor quality.

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