Phytochemistry, 1975, Vol 14, pp 2569–2572 Pergamon Press Printed in England

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE LIPOXYGENASE FROM GRAINS OF *HORDEUM DISTICHUM*

SEIZO YABUUCHI and MIKIO AMAHA

Central Research Laboratories, Asahi Breweries, Oota-ku, Tokyo 143, Japan

(*Received* 11 *March* 1975)

Key Word Index—*Hordeum distichum*, Gramineae, barley, embryo, lipoxygenase, linoleic acid, linoleic acid hydroperoxide.

Abstract—Lipoxygenase activities in ungerminated and germinating barley grains were found to be associated exclusively with the embryos. A lipoxygenase was extracted from ungerminated embryos and partially purified by fractional precipitation with ammonium sulfate and gel-filtration. Both the crude extracts and the purified preparation appeared to contain only a fatty acid type lipoxygenase which mainly converted linoleic acid to 9-hydroperoxy, *trans*-10, *cis*-12-octadecadienoic acid. The purified enzyme was inhibited by its own products, hydroperoxides, but not by 1 mM cyanide, EDTA, Hg^{2+} or Cu^{2+}

INTRODUCTION

Lipoxygenase (E.C. 1.13.1.13) which catalyses the aerobic oxidation of unsaturated fatty acids with a *cis*-1, *cis*-4-pentadiene system to conjugated *cis*, *trans*-diene hydroperoxides, has been found in a wide variety of higher plants, especially legume seeds [1–4] and cereal grains [5–7]. Recent studies have shown that volatile carbonyl compounds are formed from unsaturated fatty acids by a sequence of enzyme reactions involving lipoxygenase [8,9]

The presence of lipoxygenase in barley grains was first reported by Franke and Frehse [5]. They found that the barley enzyme differed from soybean lipoxygenase in several respects, e.g. the pH optimum. In their report, however, the reaction products of barley lipoxygenase were not characterized. Graveland *et al.* [10] showed that when incubated in barley-flour suspensions, linoleic acid was converted to two isomeric hydroperoxides and other compounds such as α-hydroxy-keto acids, keto-dihydroxy acids and trihydroxy acids. The exact pathways for the formation of these compounds have not yet been established.

To determine the mechanism of formation of volatile carbonyl compounds during malting and brewing, a study of the pathways of linoleic acid oxidation by barley enzymes was made The present communication deals with the properties of a lipoxygenase from barley embryos

RESULTS

Lipoxygenase activity in germinating barley grains

Dehusked barley grains were incubated at 20° for 8 days and germinating samples were taken at intervals of 48 hr. The embryos and remaining endosperms from 50 grains of each sample were extracted separately. As shown in Table 1, lipoxygenase activity appeared to be concentrated exclusively in the embryos, with practically no activity in the endosperms even after germination. The lipoxygenase activity of the embryos remained almost constant for the first 4 days of germination and thereafter increased rapidly. This increase is presumably due to the *de novo* synthesis of the enzyme(s) in active tissues such as the plumule

Table 1 Lipoxygenase activity in germinating barley grains

Tissue	Time after steeping (days) 4 hr	2	4	6	8
Embryos	2·97*	3·21	3·28	6·93	5·03
Endosperms	0·06	0·00	0·06	0·03	0·00

* Units/ml

Purification of lipoxygenase from barley embryos

Purification of the lipoxygenase from crude embryo extracts was carried out by fractional precipitation with ammonium sulfate and by chromatography on Sephadex. The specific activities and yields of the enzyme at three different steps of purification are shown in Table 2. Figure 1 shows the pattern of the final gel-filtration on Sephadex G-200. Both the crude extracts and final preparation showed only one active fraction; there appeared to be no other isoenzymes when examined by chromatography on DEAE-Sephadex A50 and by polyacrylamide gel disc electrophoresis.

Some properties of the barley lipoxygenase

The purified lipoxygenase preparation had an optimum at about pH 7·5, but was inactive at pH 5 and pH 9. The enzyme oxidized both linoleic acid and linolenic acid at similar rates, but was inactive with oleic acid and stearic acid. It showed no activity with esterified derivatives of linoleic acid.

The activity of our preparation from barley embryos was not inhibited by 1 mM cyanide, EDTA, Hg^{2+} or Cu^{2+} when preincubated with each of these substances for 30 min at 30° in 0·1 M tris buffer, pH 7·5 [11,12]. Ca^{2+} reported to stimulate the lipoxygenases from barley [5] and soybean [13], had no effect on our preparation. However, the enzyme's activity was remarkably inhibited by the presence of its reaction products (hydroperoxides), as shown in Fig. 2. It seems that if barley lipoxygenase plays an important physiological role *in vivo*, there must be other enzymes that decompose the hydroperoxides. When incubated in 0·1 M Pi buffer (pH 6), 80% of the lipoxygenase activity was lost after 60 min at 60°, but there was no loss in activity after 60 min at 45°. An aqueous solution of the enzyme was fairly stable at room temperature and was resistant to both freezing and freeze-drying.

Table 2 Purification of lipoxygenase from barley

Purification stage	Specific activity (units/mg protein)	Total activity (units)	Yield (%)
Extracts	2·11	1290	100
Sephadex G-150 (first)	5·20	790	61
Sephadex G-150 (second)	13·1	550	43
Sephadex G-200	24·0	240	19

Oxidation products from linoleic acid due to barley lipoxygenase

Chemical structures of the oxidation products from linoleic acid were investigated with the aid of UV, IR and MS. A reaction mixture (50 ml) containing 70 mg of linoleic acid and 26·6 units of the purified lipoxygenase preparation was incubated at 25° for 30 min with continuous shaking. After acidification to pH 2, the mixture was extracted with isooctane–*n*-butanol (19 : 1). The UV spectrum of the extracts showed the maximum absorption at 234 nm, indicating the presence of the conjugated diene structure. The yield of hydroperoxides from linoleic acid was estimated as about 83% from the absorption at 234 nm. The IR spectrum of the hydroperoxides fraction which was separated from preparative TLC on silicic acid showed a paired absorption at 943

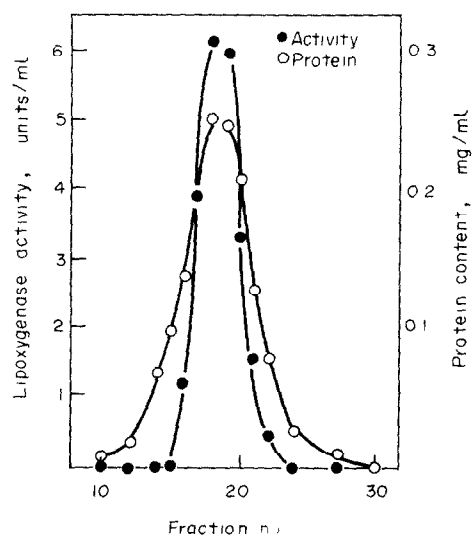

Fig. 1

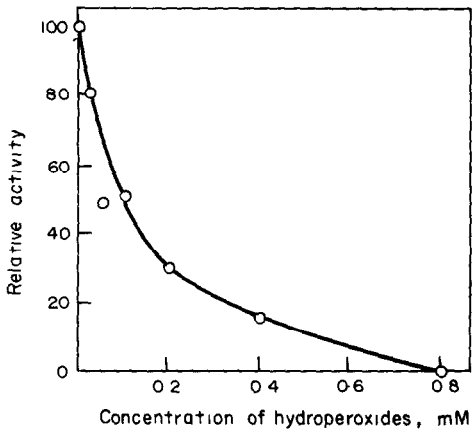

Fig 2

and 980 cm^{-1} which is characteristic of the *cis-trans* conjugated diene. The ratio of the heights of the 2 peaks was approximately 1 : 1. After esterification and hydrogenation, the hydroperoxides were subjected to GC–MS analysis. Fragment ions appeared at *m/e* 155, 158 and 187, which correspond to methyl 9-hydroxystearate, and at *m/e* 211, 214 and 243, which correspond to methyl 13-hydroxystearate. The ratio of the 9- and 13-isomers as estimated from the relative intensities of the three peaks of each isomer, was *ca* 9 : 1.

From these results, we concluded that the main oxidation product from linoleic acid due to barley lipoxygenase is 9-hydroperoxy, *trans*-10, *cis*-12-octadecadienoic acid.

DISCUSSION

Our lipoxygenase preparation from barley embryos converted linoleic acid almost exclusively (90%) into 9-hydroperoxy, *trans*-10, *cis*-12-octadecadienoic acid. This agrees with the result of Graveland *et al.* [10] who analyzed the hydroperoxides produced from linoleic acid in barley-flour suspensions. The small amount of the 13-isomer formed is possibly due to autoxidation occurring during incubation and extraction procedures.

It has been shown by other workers [3,14] that soybeans contain an ester lipoxygenase as well as a fatty acid lipoxygenase. However, in the present investigation, ungerminated barley grains appeared to contain only one type of lipoxygenase which corresponded to the fatty acid lipoxygenase. Moreover, there seemed to be no isozymes when examined with disc electrophoresis and gel-filtrations.

A preliminary study on the lipid breakdown in germinating barley grains indicated that free fatty acids appeared in the embryos after 2 days of germination, suggesting that the action of lipoxygenase *in vivo* would probably take place after this stage of germination. On the other hand, the development of some volatile carbonyls during malting has been observed after the 3rd to 5th day where the rootlet and the plumule have already emerged from the embryo. To establish the mechanisms of formation of these volatile carbonyls, it is necessary to investigate the exact nature of each of the enzymes involved in lipid breakdown at each stage in the germination of barley grains. Results of our investigation of the second enzyme, hydroperoxide isomerase, will be described in a subsequent communication.

EXPERIMENTAL

Plant material A single lot of grains of 2-row barley, *Hordeum distichum* var Satsuki Nijo (1971 harvest) was used

Germination and separation of tissues Barley grains were dehusked by treatment with 50% (v/v) H_2SO_4 for 3 hr at room temp [15] After washing with H_2O, the dehusked grains were surface-sterilized with 0 1% benzalkonium chloride sol and rinsed several times with sterile H_2O 100 grains were allowed to germinate at 20° on moistened filter paper in a 9 cm Petri dish containing 4 ml of H_2O Germinated grains were dissected into embryos and endosperms

Extraction of the enzyme Separated tissues from 50 grains were immediately homogenized in 7 ml of H_2O in a mortar until the suspensions appeared homogeneous (5–10 min) Suspensions were then centrifuged for 10 min at 10000 *g* and the supernatant was used as the crude enzyme extract

Enzyme assay Lipoxygenase activity was determined using an O_2 electrode Unless otherwise stated, incubation mixtures (10 ml) contained 10 μmol of linoleic acid or other substrates (99% pure) homogenized with 0 1 M tris buffer, pH 7 5 Mixtures were continuously stirred and after attaining equilibrium at 30°, the enzyme soln was injected Decrease in dissolved O_2 was followed for 2–3 min and the enzyme activity was calculated from the initial rates of O_2 uptake, assuming an initial dissolved O_2 concn of 0 24 mmole/l One unit of lipoxygenase corresponds to an uptake of 1 μmol of O_2 per min

Enzyme purification Embryos from 2500 ungerminated grains were thoroughly homogenized in 80 ml of H_2O Extracts were centrifuged for 10 min at *ca* 10000 *g* $(NH_4)_2SO_4$ was added to the supernatant with stirring. The ppt between 30 and 60% satn were collected and applied to a Sephadex G-150 column, which had previously been equilibrated with 10 mM Na Pi buffer, pH 6 8, containing 0 1 M NaCl Fractions of 7 ml were collected and assayed for protein by the method of Lowry *et al* [16] Fractions containing lipoxygenase activity were combined and brought to 60% satn with $(NH_4)_2SO_4$ and

again the ppt were applied to the same column After repeating the treatment, the ppt were applied to a Sephadex G-200 column under similar conditions Fractions containing lipoxygenase activity were pooled and stored in a refrigerator

Disc gel electrophoresis Electrophoresis of the enzyme was made on 7.5% polyacrylamide gels at pH 7.5, using the method of ref [17] Runs were carried out at 4° for about 60 min at 2 mA per tube Staining for enzyme activity was made by the method of ref [3]

Characterization of enzymatic oxidation products The UV spectrum was recorded with a recording spectrophotometer The hydroperoxides were chromatographed on 2 mm layers of silicic acid and were visualized using KI-starch reagent applied to separate lanes The solvent mixture was petrol Et_2O (3 2) After development, the hydroperoxides were extracted with isooctane *n*-butanol (19 1), and these were applied to a NaCl plate to prepare a liquid film for IR analysis A portion of the hydroperoxides was esterified with CH_2N_2 then hydrogenated using the method of Hamberg and Samuelsson [18] GC–MS was performed with a 200 cm column containing 1% OV-1 on Chromosorb W at an ionization potential of 70 eV and an ion source temp of 270°

Acknowledgements – We thank Mr Y Takahashi, President of Asahi Breweries Ltd., for permission to publish this study

REFERENCES

1 Theorell, H Holman R T and Akeson, A (1947) *Acta Chem Scand* **1**, 571
2 Christopher J Pistorius, E and Axelrod B (1970) *Biochim Biophys Acta* **198**, 12
3 Verhue, W M and Francke, A (1972) *Biochim Biophys Acta* **284**, 43
4 Haydar, M and Hadziyev, D (1973) *J Sci Food Agr* **24**, 1039
5 Franke, W und Frehse, H (1953) *Hoppe-Seylers Z Physiol Chem* 295, 333
6 Wallace J M and Wheeler, E L (1972) *Cereal Chem* **49**, 92
7 Gardner, H W (1970) *J Lipid Res* **111**, 311
8 Jadhav, S., Singh, B and Salunkhe, D K (1972) *Plant Cell Physiol* **13**, 449
9 Hatanaka, A and Harada T (1973) *Phytochemistry* **12**, 2341
10 Graveland, A., Pesman L and Eerde, P (1972) *MBAA Tech Quarterly* **9**, 98
11 Tappel, A L (1963) *The Enzymes*, vol 8 p 275 Ed by Boyer, P D., Lardy, H and Myrback K Academic Press, New York
12 Gallhard T and Phillips D R (1971) *Biochem J* **124**, 431
13 Restrepo, F., Snyder, H E and Zimmerman, G L (1973) *J Food Sci* **38**, 779
14 Koch, R B., Stern, B and Ferrari C G (1958) *Arch Biochem Biophys* **78**, 165
15 Pollock J R A., Kirsop, B H and Essery R E (1955) *J Inst Brew* **61**, 301
16 Lowry O H Rosebrough N J., Farr A L and Randall R T (1951) *J Biol Chem* **193**, 265
17 Williams, D E and Reisfeld R A (1964) *Ann New York Acad Sci* **121**, 373
18 Hamberg, M and Samuelsson, B (1967) *J Biol Chem* **242**, 5329