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Purification of a lipoxygenase from ungerminated barley

Characterization and product formation

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Lipoxygenase was purified from ungerminated barley (variety 'Triumph'), yielding an active enzyme with a pI of 5.2 and a molecular mass of approximately 90 kDa. In addition to the 90 kDa band SDS-PAGE showed the presence of two further proteins of 63 kDa. Western blot analysis showed cross-reactivity of each of these proteins with polyclonal antisera against lipoxygenases from pea as well as from soybean, suggesting a close immunological relationship. The 63 kDa proteins appear to be inactive degradation products of the active 90-kDa enzyme. This barley lipoxygenase converts linoleic acid mainly into (9*S*)-(10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid, and arachidonic acid into (5*S*)-(6*E*,8*Z*,11*Z*,14*Z*)-5-hydroperoxy-6,8,11,14-eicosatetraenoic acid.

Lipoxygenase; Linoleic acid hydroperoxide; Arachidonic acid hydroperoxide; Barley

1. INTRODUCTION

Lipoxygenases (linoleate:oxygen oxidoreductase EC 1.13.11.12) are a group of enzymes containing non-heme iron which catalyse the regio- and stereoselective dioxygenation of polyunsaturated fatty acids with a (*Z*, *Z*)-1,4-pentadiene system. The resulting products are optically active, conjugated (*Z*, *E*)-diene hydroperoxy derivatives.

The enzyme is found both in plants and in animals [1–4]. Its occurrence in higher plants is of special interest to food science since it converts polyunsaturated fatty acids into hydroperoxides which may decompose to form products which can have characteristic tastes and smells (both desirable and undesirable) [5]. This degradation process can be either enzymatic, as has been shown in potato [6,7], or non-enzymatic [8,9]. Two of the possible degradation products formed from 9-hydroperoxy-octadecadienoic acid are 9-oxononanoic acid and *trans*-2-nonenal. For example the latter has been shown to be mainly responsible for the occurrence of a stale flavor in beer upon storage [10].

In barley, lipoxygenase was first reported by Kolesnikov [11] in 1950, while some properties of crude enzyme preparations were later characterized [12–15]. The occurrence of a second lipoxygenase isoenzyme after germination was reported [16,17].

The object of the present work is to further characterise the barley lipoxygenase.

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2. MATERIALS AND METHODS

Barley (*Hordeum distichum*), varieties 'Blenheim' and 'Triumph' (1989 harvest), were kindly donated by A. Doderer of Heineken Technisch Beheer (Zoeterwoude, NL).

CM-Sephadex C-50, DEAE-Sepharose CL-6B and Sephacryl S-300 were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Linoleic acid (99%) was from Fluka A.G. (Buchs, Switzerland), 1-naphthoylchloride (>98%) from Aldrich Chemical (Milwaukee, WI, USA). Molecular mass standards and 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol (Bistris) buffer were from Sigma (St. Louis, MO, USA). Methanol, *N*-hexane (Merck, Darmstadt, FRG) and tetrahydrofuran (THF) (J.T. Baker, Deventer, NL) were all of HPLC grade.

Whole barley grains (300 g) were ground in an overcross beat mill (Peppink, Amsterdam) with solid carbon dioxide and extracted 3 times with 1 l of cold acetone. The resulting acetone powder was dried under vacuum and extracted with 1 l of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl at 4°C for 1 h. The extract was centrifuged (4200 × *g* for 30 min at 4°C) and the resulting supernatant is referred to as crude extract.

Lipoxygenase activity was determined polarographically using a thermostated Clark-type oxygen electrode (Hansatec, King's Lynn, UK) with air saturated reagents at 25°C. Sodium phosphate buffer (1.0 ml, 0.1 M pH 7.0) was added to the measuring chamber and stirred until equilibrated at 25°C. Then 10 μl of a linoleic acid stock solution (180 mM linoleic acid in 1 M ammonia) was added, and the reaction was started by addition of the enzyme. Decrease in O_2 concentration was followed for 2–3 min and the enzyme activity was calculated from the initial rate of O_2 uptake, assuming an initial O_2 concentration of 240 μM. One unit of lipoxygenase activity corresponds to the uptake of 1 μmol of O_2 per min.

Purification of the crude extract was performed as follows: solid ammonium sulfate was added to the crude extract and the fraction which precipitated between 30% and 60% saturation at 4°C was collected by centrifugation and dialysed for 16–20 h against 50 mM sodium acetate buffer (pH 4.7). The dialysate was centrifuged (30 min, 48 000 × *g* at 4°C) to remove precipitated material, and then applied to a CM-Sephadex C50 column (3.0 × 20 cm) equilibrated with the same buffer. Bound protein was eluted from the column with a

linear gradient of 0–300 mM NaCl in this buffer, and the active fractions were pooled and then precipitated using ammonium sulfate to 80% saturation. After resuspending the protein in 50 mM Bistris-HCl buffer (pH 6.6) it was applied to a Sephacryl S-300 gelfiltration column (2.0 × 95 cm) equilibrated with the same buffer. Active fractions were pooled and applied directly to a DEAE-Sepharose CL-6B column (2.1 × 15 cm) equilibrated with Bistris-HCl buffer (pH 6.6). Bound protein was eluted using a linear gradient of 0–300 mM NaCl in this buffer and fractions containing lipoxygenase activity were pooled and stored at 4°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% slab-gels with the Mini-protean II apparatus (Bio-Rad, Rockville Centre, NY, USA) using the method of Laemmli [18]. Runs were carried out for 45 min at 200 V. Gels were stained for 30 min with Coomassie blue R-250 (0.1% (w/v) in 40% methanol, 10% acetic acid (v/v)), and destained in 10% methanol, 7.5% acetic acid (v/v) for several hours.

Blotting on nitrocellulose membranes was done with the Mini Trans-blot cell (Bio-Rad) according to instructions of the manufacturer. The electrophoretic transfer was for 1 h at 100 V. Immune detection was performed using the Immuno-blot assay kit (Bio-Rad) with polyclonal antibodies raised against purified soybean or pea lipoxygenase.

Preparative isoelectric focussing (IEF) was performed, using the Rotofor apparatus (Bio-Rad) according to the instructions of the manufacturer, in a 2% solution of Bio-lyte pH 3–10 ampholytes. Focussing was performed for 6 h at 12 W constant power at 4°C.

Reaction products were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC). Substrate (linoleic acid or arachidonic acid) was suspended in 50 ml of oxygen-saturated 0.1 M sodium phosphate buffer (pH 7.0) to a final concentration of 200 μM, and incubated for 1 h at room temperature after addition of enzyme. The reaction was stopped by lowering the pH to 3 with dilute HCl and the products were extracted with octadecyl reversed-phase extraction columns (J.T. Baker, Deventer, NL) according to Verhagen [19], with the modification that EDTA was left out. The products were analysed on a Hypersil-C18 column (5 μm, 4.6 × 100 mm) (Hewlett Packard) using an ultraviolet detector (Kratos Spectroflow 783, set at 237 nm for detection of conjugated dienes) and an integrator (Shimadzu CR3A). Isocratic elution was performed with THF/methanol/water/acetic acid (25:30:44.9:0.1, brought to pH 5.5 with concentrated ammonia) [20] at a flow rate of 0.9 ml/min. Standards of (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD) and (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) were made by incubating linoleic acid with lipoxygenase 1 (13-HPOD) or lipoxygenase 2 (9-HPOD) from soybean at pH 9.0 [21], while standards of (6*E*,8*Z*,11*Z*,14*Z*)-5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) were made by incubating arachidonic acid with potato lipoxygenase at pH 6.3 [22].

To determine the configuration, the reaction products were reduced with $SnCl_2$ to the corresponding hydroxy fatty acids (9-HOD or 5-HETE) and subsequently converted into the methyl ester by treatment with an excess of ethereal diazomethane for 5 min at room temperature. The methyl ester was derivatized by reaction with 1-naphthoylchloride and purified by RP-HPLC as described by Hawkins [23]. The derivatized products were analyzed on a (*R*)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine (DNBPG) chiral stationary phase column (5 μm, covalently linked, 4.6 × 250 mm) (J.T. Baker) using the same detector and integrator as above. The hydroxy fatty acid derivatives were eluted isocratically with *N*-hexane containing 0.5% 2-propanol (v/v) at a flow rate of 0.8 ml/min.

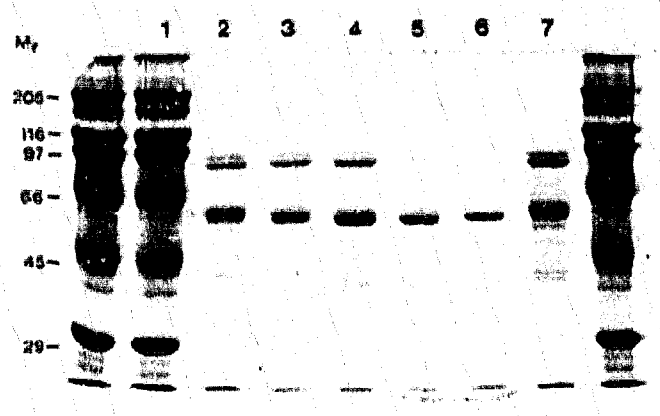

Fig. 1. 10% SDS-PAGE of samples of barley lipoxygenase stored at room temperature. Active, highly purified lipoxygenase from barley variety 'Triumph' was stored at room temperature and samples for SDS-PAGE were taken at regular intervals. The bands at approximately 90 kDa contain the active enzyme. Lane 1, molecular mass markers: myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, albumin (bovine serum) 66 kDa, ovalbumine 45 kDa, carbonic anhydrase 29 kDa. Lanes 2 and 7, barley lipoxygenase starting material. Lane 3–6, barley lipoxygenase kept at room temperature for 1, 3, 6 and 13 days, respectively.

3. RESULTS AND DISCUSSION

3.1. *Purification of lipoxygenase from ungerminated barley*

Chromatography of crude extracts of the barley variety 'Triumph' on CM-Sephadex shows only one active peak. Precipitation of this enzyme by ammonium sulfate, followed by ion-exchange chromatography and gelfiltration results in a 450-fold purification (Table I). On SDS-PAGE this preparation shows 4 bands of approximately equal intensity, two with an apparent molecular mass of approximately 90 kDa and two of about 63 kDa. This purified enzyme preparation is

Table I

Summary of purification of lipoxygenase from ungerminated barley variety 'Triumph'

Purification step	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	8488	0.52	-	100
30–60% $(NH_4)_2SO_4$	1400	2.31	4.4	73.2
After CM-Sephadex	251	10.5	20.3	59.9
After gelfiltration	45.6	67.9	131	70.0
After DEAE-Sepharose	3.2	232	448	17.6

Ungerminated barley variety 'Triumph' (300 g) was ground and the resulting flour was defatted by extraction with cold acetone. Lipoxygenase was extracted with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride.

quite stable at 4°C, but at room temperature a 50% loss of activity is noted after 60 h. SDS-PAGE of samples taken at intervals shows a gradual loss of the 90 kDa bands, while simultaneously the bands at 63 kDa become more pronounced (Fig. 1). Buffers used are filter-sterilized. Upon storage at 4°C no degradation of the 90 kDa proteins is detected after 6 weeks. The exact nature of the two 90 kDa proteins, especially the possibility of two active forms, has not yet been elucidated. At present work is being done to separate and characterize these two bands and to remove the 63 kDa proteins.

All 4 bands show cross-reactivity on Western blots with polyclonal antisera raised against lipoxygenases purified from peas or soybeans. This suggests a close immunological relationship between these lipoxygenases from different plant species, as well as between the proteins of 90 and 63 kDa. This also seems to indicate that these inactive 63 kDa proteins are degradation products of the active 90 kDa enzyme. The molecular mass of 90 kDa calculated from SDS-PAGE is in close agreement with the known values of 94, 96 and 97 kDa for the soybean isozymes [24], and 97 kDa for two of the pea isozymes [25,26]. Führling [15] found a mass of 64 kDa for barley lipoxygenase by means of gelfiltration, which is remarkably close to the mass found for the two inactive proteins of 63 kDa.

The enzyme exhibits a broad optimum around pH 7.0, and the pI was determined, by preparative IEF, to be 5.2, while the variety 'Blenheim' is found to also contain an isozyme with a pI of 6.9. This isozyme, which accounts for only approximately 10% of the total lipoxygenase activity in ungerminated 'Blenheim' barley, has the same pI as a lipoxygenase isolated from germinating barley by Yabuuchi [16]. Since this second isozyme is not found in extracts from the variety 'Triumph' it is concluded that different varieties of a plant species can have different isozyme contents.

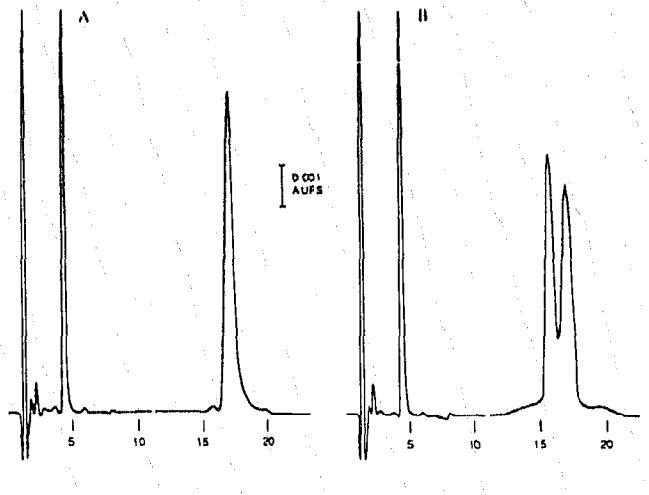

Fig. 2. RP-HPLC of products formed by incubation of linoleic acid with barley lipoxygenase at pH 7.0. Elution of 5 μm nucleosil-C18 column (4.6 × 100 mm) with THF/MeOH/H_2O/HAc (25:30:45:0.1), pH 5.5, at 0.9 ml/min, ultraviolet absorption detection at 237 nm. (A) elution pattern of products formed on incubation of linoleic acid with barley lipoxygenase at pH 7.0 (Peak at 4 min is prostaglandin B_2 used as internal reference, peak at 17 min is the product formed). (B) co-elution pattern of barley lipoxygenase products of linoleic acid and 13-HPOD standard made by incubation of linoleic acid with soybean lipoxygenase 1 at pH 9.0. (Peak at 4 min is prostaglandin B_2, peak at 16 min is 13-HPOD standard, peak at 17 min is the formed product.)

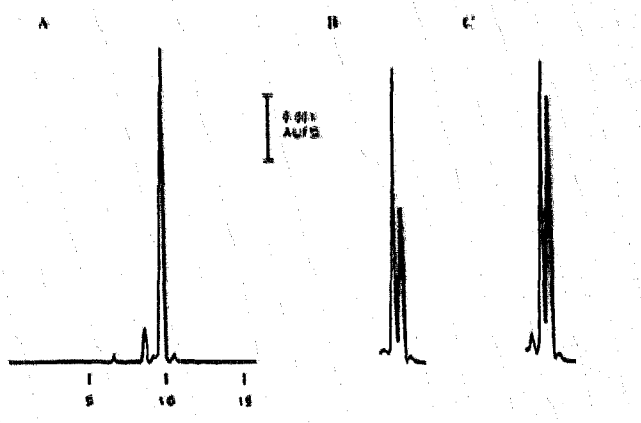

Fig. 3. Chiral Phase-HPLC of the 1-naphthoylated methyl ester of the incubation product of linoleic acid with barley dipoxygenase at pH 7.0. Elution of 5 μm Bakerbond chiral phase DNBPG column (covalent, 4.6 × 250 mm) with 0.5% 2-propanol in *N*-hexane at 0.8 ml/min, ultraviolet absorption detection at 237 nm. (A) elution pattern of derivatized product of barley lipoxygenase. (B) elution pattern of 9-HOD standards made by incubation of linoleic acid with soybean lipoxygenase 2 at pH 9.0 (ratio of *R*:*S* approximately 2:1. (C) co-elution pattern of the derivatized product and 9-HOD standards.

3.2. *Product specificity of purified barley lipoxygenase*

Products formed upon incubation of linoleic acid with the purified enzyme at pH 7.0 are analysed by RP-HPLC (Fig. 2), and it is concluded that the main product (>95%) of this barley lipoxygenase is 9-HPOD. This is in good agreement with previous work, where yields of 70% [27], 89% [28], 90% [13,29] and 96% [15] were reported for 9-HPOD. The configuration of this 9-HPOD is shown to be essentially (>97%)*S* (Fig. 3). Therefore the primary product of this barley lipoxygenase is (9*S*)-(10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid.

The enzyme can also convert arachidonic acid, although the activity is only about 15% of that found for linoleic acid as substrate. The main product of this reaction is 5-HPETE (>85%), the configuration of which is found to be *S*(>97%). Further work is being done to establish whether this enzyme, like other 5-lipoxygenases, can catalyse the formation of leukotrienes. At present no evidence for such products has been found.

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