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COMPARISON OF FATTY ACID EPOXIDE HYDROLASE ACTIVITY IN SEEDS FROM DIFFERENT PLANT SPECIES

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Key Word Index—*Arabidopsis thaliana*; *Brassica napus*; *Sinapis alba*; Brassicaceae; *Euphorbia lagascae*; *Ricinus communis*; Euphorbiaceae; *Linum usitatissimum*; Linaceae; *Nicotiana tabacum*; Solanaceae; *Glycine max*; *Pisum sativum*; *Vicia faba*; *Vicia sativa*; Fabaceae; seed; epoxide hydrolase (EC 3.3.2.3); fatty acid epoxide; *cis*-9,10-epoxystearic acid.

Abstract—Epoxide hydrolase (EC 3.3.2.3) activity was measured with *cis*-9,10-epoxystearic acid as the substrate. The activity was determined in homogenates (600 *g* supernatants) prepared from seeds after two days of germination. The species analysed were *Arabidopsis thaliana*, *Brassica napus*, *Euphorbia lagascae*, *Glycine max*, *Linum usitatissimum*, *Nicotiana tabacum*, *Pisum sativum*, *Ricinus communis*, *Sinapis alba*, *Vicia faba* and *Vicia sativa*. Fatty acid epoxide hydrolase activity was present in all species, which could be divided into two groups with low or high activity. The highest specific activity was found in *G. max* and *P. sativum*, while the highest total activity was observed in the former. Our findings indicate that fatty acid epoxide hydrolase may be a ubiquitous enzyme in plant seeds.

INTRODUCTION

Plant seeds store large amounts of proteins, lipids and carbohydrates to enable germination and subsequent development into a mature plant. In oil-storage seeds, large amounts of fatty acids in the form of triacylglycerols are used as a source of energy and biosynthetic intermediates. The composition of fatty acids is unique to each type of seed with large variations in chain length and degree of unsaturation, although a certain variation within the same species is common [1].

Epoxy fatty acids are common storage lipids in seeds of certain species. At the extreme end are members of the Asteraceae, which have been reported to have over 70% of the lipids in the form of the epoxy fatty acids in the seed [2]. Vicinal dihydroxy fatty acids are also abundant in some species, e.g. from the Asteraceae with 25% of the fatty acids in the form of diols [2]. The high levels of these fatty acid epoxides or diols indicate that certain enzymes are highly active, while other enzymes are absent or have very low activity causing an accumulation of different metabolites. The function and the metabolism of epoxy fatty acids in plants have received little attention. In animal cells though, several enzymes involved in the metabolism of epoxy fatty acids have been characterized. Cytochrome P-450 and epoxide hydrolase seem to be key enzymes in the formation and the breakdown of epoxy fatty acids, respectively [3]. In plants, several enzymes with the ability of forming fatty acid epoxides have been found but much less is known about the further metabolism of fatty acid epoxides [4]. An interesting function of epoxy- and dihydroxylinoleic acid in rice is the discovery of their fungicidal properties towards *Pyricularia oryzae* [5].

We are interested in the function and metabolism of fatty acid epoxides, as well as the properties of the enzymes involved in their metabolism in plants. In order to analyse the presence and the relative activity of fatty acid epoxide hydrolase in different plants, we have analysed seeds from 11 species both from oil-storage species and from species with low contents of seed oil.

RESULTS AND DISCUSSION

Seeds were germinated for two days to stimulate the differentiation processes, including the glyoxysomal pathways regarding β-oxidation of fatty acids and the glyoxylate cycle [6]. An earlier analysis of fatty acid epoxide hydrolase activity in *Ricinus communis* endosperm showed high activity during the initial nine days of germination analysed, with a maximum around day two (Meijer *et al.*, manuscript in preparation). The seeds were therefore germinated for two days before analysis in order to minimize the risk of low activity being overlooked. Enzyme activity was found in all tissues examined (endosperm, cotyledons, hypocotyl and root) from *R. communis* seeds, although with different specific activities. No endogenous inhibitors seemed to be present, since mixing tissues gave an additive effect. In the present investigation, the whole seed was analysed due to the extensive labour involved with microdissection of small seeds.

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Fatty acid epoxide hydrolase activity was found in the 600 *g* supernatant from all species examined. The product had the same relative migration on TLC as synthetic *threo*-9,10-dihydroxystearic acid. No other products were observed during the incubations. Boiled preparations of the 600 *g* supernatants had an activity that was lower or in the same range as the buffer blank indicating that the reaction is catalysed by an enzyme in all species.

When the epoxide hydrolase activities of the seeds were compared, two groups were distinguished, with *Vicia faba*, *Vicia sativa*, *Pisum sativum* and *Glycine max* having comparatively high specific activities (Fig. 1A). In contrast, the other seeds examined had approximately 10-fold, or even lower, specific activities. Some caution is warranted when comparing the different seeds, however, since cells from different sources may differ in their susceptibility to disruption during homogenization in the mortar. When some of the different homogenates from species with low and high activities were mixed and assayed for fatty acid epoxide hydrolase activity, the results were additive. Thus, the low activity in some samples does not seem to be due to the presence of endogenous inhibitors.

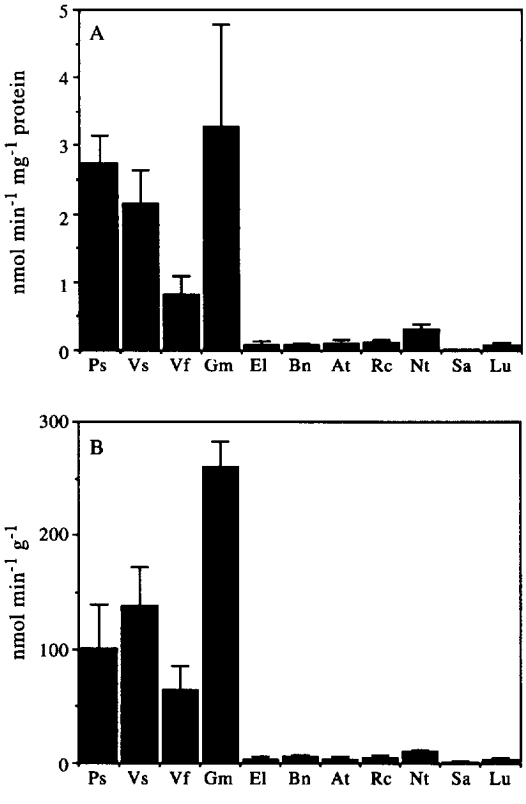

Fig. 1. Fatty acid epoxide hydrolase activity in seed homogenates after two days of germination. Activities were measured as described in the Experimental and specific activity (A) and total activity (B) are shown as mean ± s.d. of measurements performed from two or three different preparations. Ps (*Pisum sativum*), Vs (*Vicia sativa*), Vf (*Vicia faba*), Gm (*Glycine max*), El (*Euphorbia lagascae*), Bn (*Brassica napus*), At (*Arabidopsis thaliana*), Rc (*Ricinus communis*), Nt (*Nicotiana tabacum*), Sa (*Sinapis alba*) and Lu (*Linum usitatissimum*).

When the fatty acid epoxide hydrolase activity was expressed per g of seed, a similar pattern of relative differences between the seeds was observed (Fig. 1B). In the case of *Arabidopsis thaliana*, *Brassica napus* and *Nicotiana tabacum*, the seed coat is included in the weight. Accordingly, these values expressed per g of seed are lower in comparison with the other species. Relatively low fatty acid epoxide hydrolase activity was found in seeds with low or high levels of oil. Relatively high enzyme activity was found in the four plants of the Fabaceae, where *G. max* has a relatively high oil level in contrast to the other three species analysed. No simple correlation between fatty acid epoxide hydrolase activity of the seeds and their oil content could thus be made. If high fatty acid epoxide hydrolase activity is a general property of the Fabaceae, the physiological aspects need to be established as well.

Earlier reports of fatty acid epoxide hydrolase in plants have investigated the activity in the cytosolic fraction of *V. faba* [7] and *G. max* [8]. Our value for the specific activity of *G. max* fatty acid epoxide hydrolase activity was similar to the reported value of 3.4 nmol min^{-1} and mg protein for the 100 000 *g* supernatant [8]. In our case, a 600 *g* supernatant was used, which means that the protein content will be higher than in the high-speed supernatant and, accordingly, the final specific activity would be somewhat lower in our case.

The hydration of epoxy fatty acids may serve different purposes. One important function would be to produce diols with antipathogenic properties [5]. Such formation would presumably be activated by elicitors through a signal transduction pathway [9]. Another important function would simply be to bypass blocks introduced in the β-oxidation spiral by the epoxy group in fatty acids. In this case, α-oxidation may also be needed for complete β-oxidation of the fatty acid [10, 11]. The presence of fatty acid epoxide hydrolase activity in all seeds analysed indicates that the enzyme participates in important basic functions of the cells and may be a ubiquitous enzyme in plant seeds. We will further investigate to see if several forms of fatty acid epoxide hydrolase are present and determine the subcellular localization of the enzyme(s).

EXPERIMENTAL

Chemicals. Methyl 9,10-epoxystearate, oleic acid, benzamidine hydrochloride, BSA, dithiothreitol, phenylmethylsulphonyl fluoride (Sigma); 3-chloroperoxybenzoic acid, sodium azide (Merck); Trasylol (aprotinin) (Bayer); [1-^{14}C]-oleic acid (1.96 GBq ($mmol^{-1}$) (Amersham) were purchased from the sources indicated. Radioactively labelled and unlabelled oleic acid were methylated in acidic methanol before oxidation with 3-chloroperoxybenzoic acid to form methyl [1-^{14}C]-*cis*-9,10-epoxy stearate and methyl-*cis*-9,10-epoxy stearate. The corresponding diols were synthesized in dimethoxyethane–MeOH–perchloric acid (10:10:3) at room temp.

during 2 hr. The methylated products were demethylated in 6% NaOH in 80% MeOH overnight. The products were purified by extraction and, if needed, by TLC.

Assays. Fatty acid epoxide hydrolase activity was measured essentially as described in ref. [8] with some modifications. The routine assay was performed in duplicate in 0.1 M K-Pi (pH 7) at 30°. The substrate concn was kept at 100 μM and contained *ca* 50000 dpm per incubation. The fatty acid epoxide was dissolved in EtOH with a final concn of 2% solvent in the assay. Buffer blank or boiled enzyme control were always included in the assays. The incubations were terminated by the addition of MeCN and analysed by TLC on silica gel (LK5DF, Whatman) using Et_2O–*n*-hexane–HOAc (14:6:0.1). Plates were dried and the pattern analysed after overnight exposure by a phosphor imager and densitometry performed using ImageQuant software. Protein content was determined as described in ref. [12].

Prepn of subcellular frs. Seeds from *Arabidopsis thaliana* (mouse ear cress) (kind gift of Gunvor Sandman, Dept of Molecular Genetics), *Brassica napus* (oil-seed rape) and *Sinapis alba* (white mustard) (kind gift of Anders Falk, Dept of Cell Research), *Euphorbia lagascae* (kind gift of Sten Stymne, Dept of Plant Physiology), *Glycine max* (soybean) and *Vicia sativa* (Svalöf AB, Malmö, Sweden), *Nicotiana tabacum* (tobacco) (kind gift of Kjell Stålberg, Dept of Cell Research), *Ricinus communis zanzibariensis* (castor bean) (Euro-Tec AB, Stockholm, Sweden), *Pisum sativum* (pea), *Vicia faba* (broad bean) and *Linum usitatissimum* (flax) (Svalöf Weibull AB, Hammenhög, Sweden) were disinfected by immersion in 0.2% licin over night. Seeds were then rinsed with sterile H_2O and germinated in MS-2 medium at 25° in darkness for 2 days. Seeds were then rinsed in sterile H_2O and the seed coats removed (except for *A. thaliana*, *B. napus* and *N. tabacum*). All tissues present were used for subcellular fractionation. Material from several seeds was used in the experiments in order to compensate for biological variation. Seeds were kept on ice, weighed and chopped with a scalpel before grinding in a chilled mortar in homogenization buffer (0.25 M sucrose, 100 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 0.1% EtOH, 10 U ml^{-1} trasylol, 1 mM benzamidine hydrochloride and 1 mM phenylmethylsulphonyl fluoride). The slurry was diluted to 10% homogenate and filtrated through Miracloth. The resulting homogenate was centrifuged at 600 g_{av} for 5 min. All frs prepd were flushed with N_2 or Ar and stored at − 80° if not analysed at once.

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