Fatty acid oxidation by lipoxygenase and ß-oxidation in germinating barley

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Chapter 1

Introduction

INTRODUCTION

Plant seeds store food reserves prior to a period of dormancy or of unfavourable environmental conditions. These reserves are used for germination and postgerminative growth of the seedling. Principally, the three classes of storage compounds are fat/oils, carbohydrates and proteins, of which fat/oils represent the most efficient way of energy storage (Huang, 1992). Fat/oils are present mainly in the form of triacylglycerols (TAG), which are partitioned in small discrete intracellular organelles, called oil bodies (Tzen and Huang, 1992). Oil bodies, also called lipid bodies or spherosomes consist of TAGs, surrounded by a monolayer of phospholipids and oleosines (Tzen and Huang, 1992; Hills, Watson and Murphy, 1993).

Following germination TAGs are mobilized, whereby oxidation of the fatty acids may occur by at least 4 different oxidative pathways, namely α-oxidation, ß-oxidation, ω-oxidation and lipoxygenase. α-Oxidation results in the removal of one carbon unit from the carboxyl terminus of the fatty acid chain and appears to be restricted to long-chain fatty acids. Chain shortening by α-oxidation will not proceed beyond C_{12} in chain length (Gerhardt, 1992). ω-oxidation results in oxidation at the methyl-end of the fatty acid molecule, and fulfils a biosynthetic rather than catabolic function. ß-oxidation results in the removal of two carbon units at the carboxyl terminus of the fatty acid, and is the sole degradation process which may lead to complete degradation of fatty acids. The lipoxygenase pathway starts with the incorporation of oxygen in *cis*-double unsaturated fatty acids, a reaction catalyzed by the enzyme lipoxygenase. The primary reaction products, fatty acid hydroperoxides, may be metabolized, yielding products with significant physiological importance.

This thesis will deal with fatty acid oxidation in germinating barley by ß-oxidation and lipoxygenase. In this chapter, a condensed overview of both pathways will be presented.

Localization and physiological function

It is generally believed that in higher plant cells, ß-oxidation occurs solely within specific organelles, called microbodies (Kindl, 1987), although a few reports suggest a dual location in both microbodies and mitochondria (Wood *et al.*, 1986; Masterson *et al.*, 1990; Masterson *et al.*, 1992; Dieuaide *et al.*, 1993).

Microbodies exhibit a diameter from 0.2-1.7 μm, contain a coarsely granular or fibrillar matrix and have a single membrane. Since, like in animal cells, microbodies in plants contain the enzyme catalase and at least one H_2O_2-producing oxidase, they may be designated peroxisomes (Huang *et al*, 1983). On basis of different functions plant microbodies can be subcategorized into glyoxysomes, leaf peroxisomes, and other unspecialized peroxisomes (Huang *et al.*, 1983). Fatty acid ß-oxidation has been characterized to be a basic function of each microbody class. Highest activities of ß-oxidation enzymes have been detected in lipid-rich tissues (Gerhardt, 1986).

In glyoxysomes, ß-oxidation is coupled to the glyoxylate cycle, resulting in the formation of sugars (Cooper and Beevers, 1969; Gerhardt, 1986; Kindl, 1987). These organelles are commonly found in lipid-rich tissues of seeds, and play a major role in the mobilization of fat reserves. During the greening of germinating oil-seed cotyledons, a functional transition occurs, whereby glyoxysomes are transformed directly into leaf peroxisomes (Titus and Becker, 1985; Nishimura *et al.*, 1986). Leaf peroxisomes, present in green leaves and cotyledons, contain enzymes of the glycolate cycle and participate in the photorespiration process. They show ß-oxidation activity (Gerhardt 1986), but lack an active glyoxylate cycle (Kindl, 1987). However, in dark-induced senescing or naturally senescing leaves (Pistelli *et al.*, 1991) or detached darkened leaves and cotyledons (De Bellis *et al.*, 1990), glyoxylate cycle enzymes could be detected together with ß-oxidation enzymes, which is consistent with a peroxisome-glyoxysome transition. Finally, ß-oxidation activity has been detected in unspecialized peroxisomes (Macey and Stumpf, 1982; Gerhardt, 1983; Pistelli *et al.*, 1989) which are found in achlorophyllous and non-oil storage tissues. These organelles contain peroxisomal

enzymes such as catalase, glycolate oxidase and urate oxidase.

In conclusion, in fatty tissues the function of ß-oxidation is well understood, namely mobilization of fat reserves enabling sugar formation, which benefits the growth of the germinating embryo. However, the function of ß-oxidation in non-fatty tissues is not yet clear. In these tissues, where ß-oxidation is located in non-specialized peroxisomes and leaf peroxisomes, ß-oxidation may be used for generation of energy (Kindl, 1987) or may be linked to the turnover of membrane lipids or proteins (Gerhardt, 1986). Also, a role in growth of coleoptiles has been suggested (Pistelli *et al.*, 1996), while an involvement in metabolism in senescent tissues (Wanner *et al.*, 1991; Pistelli *et al*, 1995) suggests a general role of peroxisomes in the metabolism of membrane components (Pistellli *et al.*, 1996).

In studies in which both a mitochondrial and microbody location of ß-oxidation have been described, ß-oxidation in microbodies was suggested to function for gluconeogenesis and energy production, while at the mitochondrial site it may function to shorten fatty acids to mid-chain length (Masterson *et al*, 1992). This is in fact contrary to the situation in mammalian cells, where mitochondrial ß-oxidation proceeds to completion, whilst microbody ß-oxidation serves to shorten fatty acids, which are then transported (Bieber 1988) Alternatively, plant mitochondrial ß-oxidation may function during glucose starvation, a situation which results in a shift from carbohydrate metabolism to fatty acid and protein utilization (Dieuaide *et al.*, 1992). In this case, mitochondrial ß-oxidation may function as a spillover for the peroxisomes with the increased availability of substrates (Dieuaide *et al.*, 1993).

Enzymes

Peroxisomal ß-oxidation

The biochemistry of peroxisomal ß-oxidation in plants has been studied extensively (reviewed by Kindl, 1987; Gerhardt 1992). Fatty acid degradation starts with the release of a fatty acid from a structural lipid or a triglyceride (Kindl, 1987), stored in lipid bodies. This reaction is catalyzed by the enzyme lipase. ß-Oxidation of fatty acids derived from storage lipids principally occurs in glyoxysomes. ß-Oxidation encompasses activation of a fatty acid, followed by

oxidative steps and removal of acetyl-CoA, together shortening the carbon chain by a C-2 moiety (Figure 1).

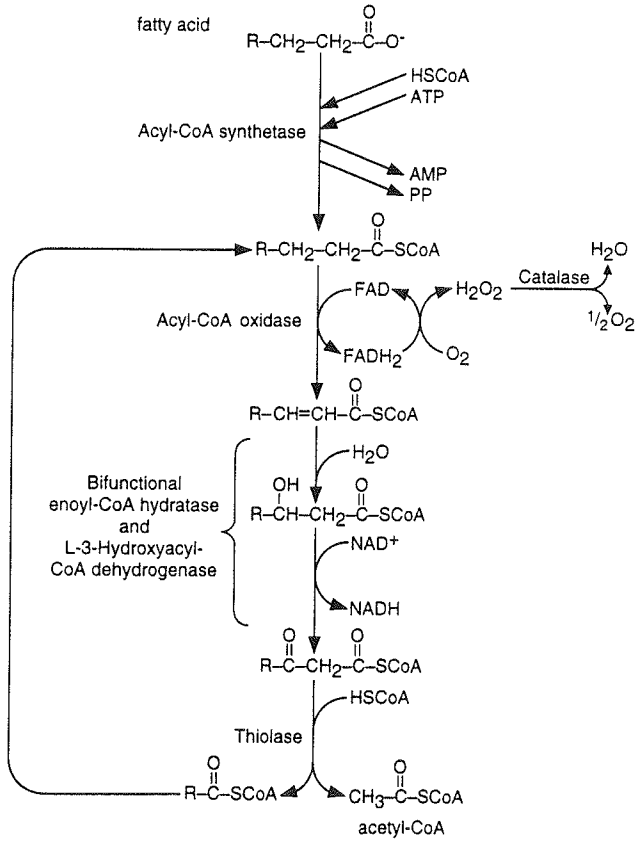

Figure 1. Peroxisomal ß-oxidation pathway in plants (adapted from Gerhardt, 1986)

The first step in the ß-oxidation pathway is energy (that is ATP) dependent and is catalyzed by the enzyme acyl-CoA synthetase. In this step, a CoA-moiety is linked to the fatty acid by the enzyme, resulting in an acyl-CoA ester. Substrate specificity studies showed that short-chain fatty acids ($\leq n=8$) are poor substrates for the enzyme (Gerbling and Gerhardt, 1987). While other ß-oxidation enzymes in higher plant peroxisomes are soluble matrix proteins, or are loosely associated with the organelle membrane (Frevert and Kindl, 1980; Gerbling and Gerhardt, 1987; Miernyk and Trelease, 1981), the acyl-CoA synthetase is tightly associated with the peroxisome membrane (Gerbling and Gerhardt, 1987).

The first oxidative step in the peroxisomal ß-oxidation reaction sequence is catalyzed by the enzyme acyl-CoA oxidase which oxidizes the acyl-CoA to 2-*trans*-enoyl-CoA. Electrons are transferred to FAD to form $FADH_2$, which is then autoxidized by molecular oxygen to generate hydrogen peroxide. This toxic component is degraded by the enzyme catalase, which is regarded as a marker of peroxisomes, and shows an extremely large catalytic turnover of hydrogen peroxide (Huang, 1983).

The next two steps in peroxisomal ß-oxidation, i.e., the hydration of 2-*trans*-enoyl-CoA to 3-hydroxyacyl-CoA and the subsequent oxidation of 3-hydroxyacyl-CoA to 3-oxoacyl-CoA are catalyzed by a bifunctional protein, for which NAD serves as an electron acceptor.

The last step in the peroxisomal ß-oxidation is catalyzed by the enzyme thiolase, which cleaves 3-oxoacyl-CoA (C_n) to acyl-CoA (C_{n-2}) and acetyl-CoA. Based on turnover data, it was suggested that the steps catalyzed by acyl-CoA oxidase and thiolase are the rate limiting steps in ß-oxidation (Kindl, 1987)

Since most plants are rich in *cis*-unsaturated fatty acids, additional enzymes are necessary for the degradation of these compounds. When a 3-*cis*-enoyl-CoA is formed during chain shortening, only an isomerase is required which converts 3-*cis*-enoyl-CoA into a *trans*-2-enoyl-CoA, which can be degraded further (Kindl, 1987). However, when a 4-*cis*-enoyl-CoA is formed, not only an isomerase is required, but also an D-3-hydroxyacyl-CoA-converting 2-enoyl-CoA hydrolyase (Engeland and Kindl, 1991). Both monofunctional enzymes and multifunctional proteins are involved in this process (Gühnemann-Schäfer and Kindl, 1995).

Mitochondrial ß-oxidation

In mammalian systems, ß-oxidation occurs both in microbodies and mitochondria (Bremer and Osmundsen, 1984; Mannaerts and Van Veldhoven, 1991; Schulz, 1991). For plants, it has been a long-standing issue whether ß-oxidation exists in mitochondria, whereby mitochondrial ß-oxidation activity often was attributed to contamination with microbodies. Doubt about ß-oxidation in higher plant mitochondria was raised by several unsuccessful attempts to detect acyl-CoA dehydrogenase activity. This enzyme catalyzes the first oxidative step in mitochondrial ß-oxidation, being the counterpart of acyl-CoA oxidase in microbody ß-oxidation. However, detection of the enzyme in maize roots after glucose starvation, indicates that at least under certain conditions mitochondrial ß-oxidation may exist also in plants (Dieuaide *et al.*, 1993). Electrons generated in this step are channeled via FAD into the mitochondrial electron transport chain. As a consequence, mitochondrial ß-oxidation is sensitive to inhibitors of the electron transport chain such as cyanide, in contrast to peroxisomal ß-oxidation. Another difference between mitochondrial and microbody ß-oxidation concerns the requirement of a carnitine acyl-transferase, which is necessary to facilitate transport of the fatty acid across the mitochondrial membrane (Gerbling and Gerhardt, 1988; Thomas and Wood, 1986; Masterson *et al.*, 1992). Finally, each activity in mitochondrial ß-oxidation is attributed to individual proteins, whereas in peroxisomes multifunctional proteins are involved in ß-oxidation.

The lipoxygenase reaction

Lipoxygenase catalyzes the incorporation of molecular oxygen into polyunsaturated fatty acids containing a 1*Z*,4*Z*-pentadiene structure (Figure 2). In plants, the most abundant fatty acids containing such a structure are linoleic acid and α-linolenic acid, and the primary reaction products of the lipoxygenase reaction are monohydroperoxides containing a *Z*,*E* conjugated diene (Vick, 1993).

The conjugated double bond in the reaction product enables spectrophotometric detection of the enzyme, because of strong absorption at 234 nm by the conjugated double bond system in the product. Alternatively, activity can be followed by monitoring molecular oxygen uptake with use of an oxygen-specific electrode. The regio-specificity, i.e., the specific site of oxygen attack, is dependent upon the source of the enzyme and upon the reaction conditions. So, with linoleic acid either 9- or 13-hydroperoxide derivatives may be formed as the products (reviewed by Vick and Zimmermann, 1987; Vick, 1993). Several plants, such as soybean, potato, rice, pea and cucumber, contain different lipoxygenase isoenzymes (Rosahl, 1996), with various regio-specificity.

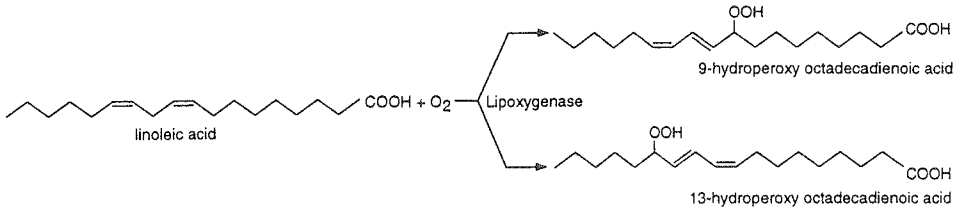

Figure 2. The primary reaction catalyzed by lipoxygenase using linoleic acid (9*Z*,12*Z*-octadecadienoic acid) as a substrate, showing the two possible reaction products, 9- and 13-hydroperoxy octadecadienoic acid (adapted from Siedow, 1991)

Subcellular location of lipoxygenases

Many attempts have been made to elucidate the subcellular location of lipoxygenase forms in plants, but often with conflicting results. Many reports concluded that lipoxygenases are predominantly soluble enzymes, present in the cytoplasm (e.g. Vernooy-Gerritsen *et al.*, 1983; Vernooy-Gerritsen *et al.*, 1984). However, lipoxygenases have also been demonstrated to be associated with chloroplasts in green leaves (Douillard and Bergeron, 1981), with lipid bodies in etiolated cotyledons (Feussner and Kindl, 1994), with microsomes of senescent tissue (Todd *et al.*, 1990) and with vacuoles of roots and fruits (Wardale and Galliard, 1977).

Physiological functions of lipoxygenase reaction products

The function of lipoxygenases in mammalian cells is well understood, namely a role in the biosynthesis of regulatory molecules, which are involved in mediating inflammatory responses (Samuelsson *et al.*, 1987). In contrast, the role of lipoxygenases in plants is not yet clear. Several physiological functions have been suggested for plant lipoxygenases, such as a role in plant growth and development, senescence, plant-pathogen interaction, lipid mobilization and the biosynthesis of regulatory molecules.

More insight into these functions may be obtained by studying the fate of the reaction products of the lipoxygenase reaction. The primary reaction products, fatty acid hydroperoxides, can be degraded in several metabolic pathways, leading to the formation of compounds of significant physiological importance, such as odors, jasmonates and oxolipins. Two pathways, namely those starting with the enzymatic action of lyase (Galliard and Philips, 1976; Vick and Zimmermann, 1976) and allene oxide synthase (Hamberg, 1987; Brash *et al.*, 1988) have been well characterized. Recently, two additional pathways have been suggested by which fatty acid hydroperoxides may be degraded, the peroxygenase pathway (Blée and Schuber, 1993) and the reductase pathway (Feussner *et al.*, 1997).

In the continuation of this section, the above mentioned fatty acid hydroperoxide-degrading pathways will be briefly described and will be related to possible roles of

lipoxygenases.

The hydroperoxide lyase pathway

In the lyase pathway, hydroxyperoxides of linoleic and linolenic acids are converted into aldehydes and ω-oxoacids (Galliard and Phillips, 1976; Vick and Zimmermann, 1976) (Figure 3).

Both reaction products of linoleic acid, 9-hydroperoxy octadecadienoic (9-HPOD) and 13-hydroperoxy octadecadienoic (13-HPOD), are converted into 3*Z*-nonenal and 9-oxo-nonanoic acid, and *n*-hexanal and 12-oxo-9*Z* dodecenoic acid, respectively (Figure 3). In most plants, the 3*Z*-enal structure is quickly isomerized to the 2*E*-enal form (Phillips *et al.*, 1979). Thus, 9-HPOD yields 2*E*-nonenal and 9-oxo-nonanoic acid, and 13-HPOD yields hexanal and 12-oxo-10*E*-dodecenoic acid.

The hydroperoxide lyase pathway has been suggested to be involved in plant responses to wounding and other plant related stresses (Galliard, 1978). For instance, 2*E*-hexenal, a hydroperoxide lyase product originating from linolenic acid is an effective fungicide (Zeringue and McCormick, 1989), bactericide (Schildknecht and Rauch, 1961) and insecticide (Lyr and Banasiak, 1983). Furthermore, 12-oxo-10*E*-dodecenoic acid, also called traumatin or wound hormone, has been suggested to mimic physiological effects upon wounding of plant tissue, including cell division and callus formation (Zimmermann and Coudron, 1979), which supports a role of hydroperoxide lyase as protective plant enzyme.

The aldehyde products of the lyase pathway include odors commonly associated with the plants which produce them (Gardner, 1991) and which are important components of flavor and aromas of leaves and fruit (Gardner, 1985; Phillips and Galliard, 1987). For instance, hexanal, 3*Z*-nonenal and 2*E*-nonenal are components of the characteristic flavor and odor of cucumber (Vick and Zimmerman, 1987). However, the volatile aldehydes are also held responsible for undesirable flavor and detoriation of foods, and are in particular associated with raw legumes such as

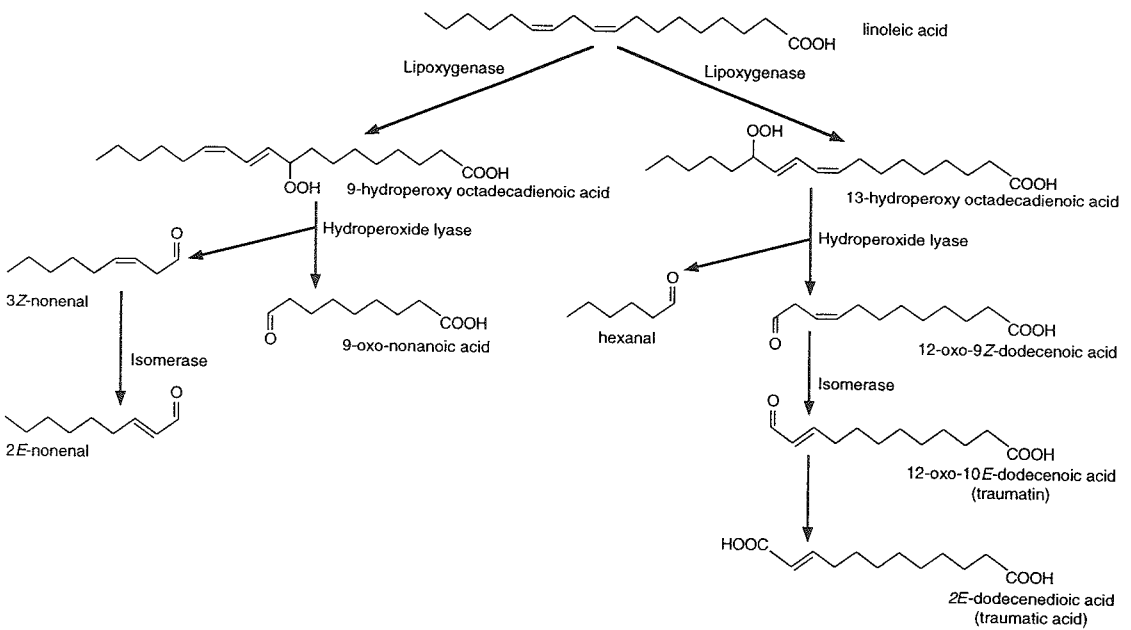

Figure 3. Production of lipoxygenase metabolites via the hydroperoxide lyase pathway (adapted from Van Aarle, 1993)

pea (Lee and Wagenknecht, 1958), lentil and soybean (Kon *et al.*, 1970). One of the aldehyde products, 2*E*-nonenal, has been identified as the compound responsible for the typical cardboard flavor in stale beer (Jamieson and Van Gheluwe, 1970). Under certain conditions, alcohol dehydrogenase can convert the aldehyde products to their corresponding alcohols. The alcohol product 3*Z*-hexenol is also known as leaf alcohol and contributes to the aroma of green leaves (Vick and Zimmermann, 1987).

The allene oxide synthase pathway

A second fatty acid hydroperoxide-degrading pathway starts with the action of allene oxide synthase, previously known as hydroperoxide dehydrase, which results in the formation of unstable allene oxide products from fatty acid hydroperoxides (Hamberg, 1987)(Figure 4).

With 13(S)-HPOT as a substrate, allene oxide synthase produces 12,13(S)-epoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid. This allene oxide can be converted non-enzymatically into α-ketols and γ-ketols. Under certain conditions, spontaneous cyclization of the allene oxide products occurs to form racemic 12-oxo-phytodienoic acid. Alternatively, the allene oxide can be metabolized enzymatically, leading to the biosynthesis of jasmonic acid. Jasmonate is a plant signalling molecule and may cause abscission, senescence and inhibition of growth (Sembdner and Pathier, 1993; Bell *et al.*, 1995), induction of leaf proteins (Staswick *et al.*, 1991), or responses to wounding and pathogen attack (Creelman *et al*, 1992; Farmer and Ryan, 1992). No specific function of the ketol products is known. It has been suggested that in wounded plants an environment is created more favorable for non-enzymatic conversion of allene oxides (Vick, 1993). Alternatively, ketol formation may occur as a protective measure in situations where an excess of fatty acid hydroperoxides has to be converted (Vick and Zimmerman, 1987).

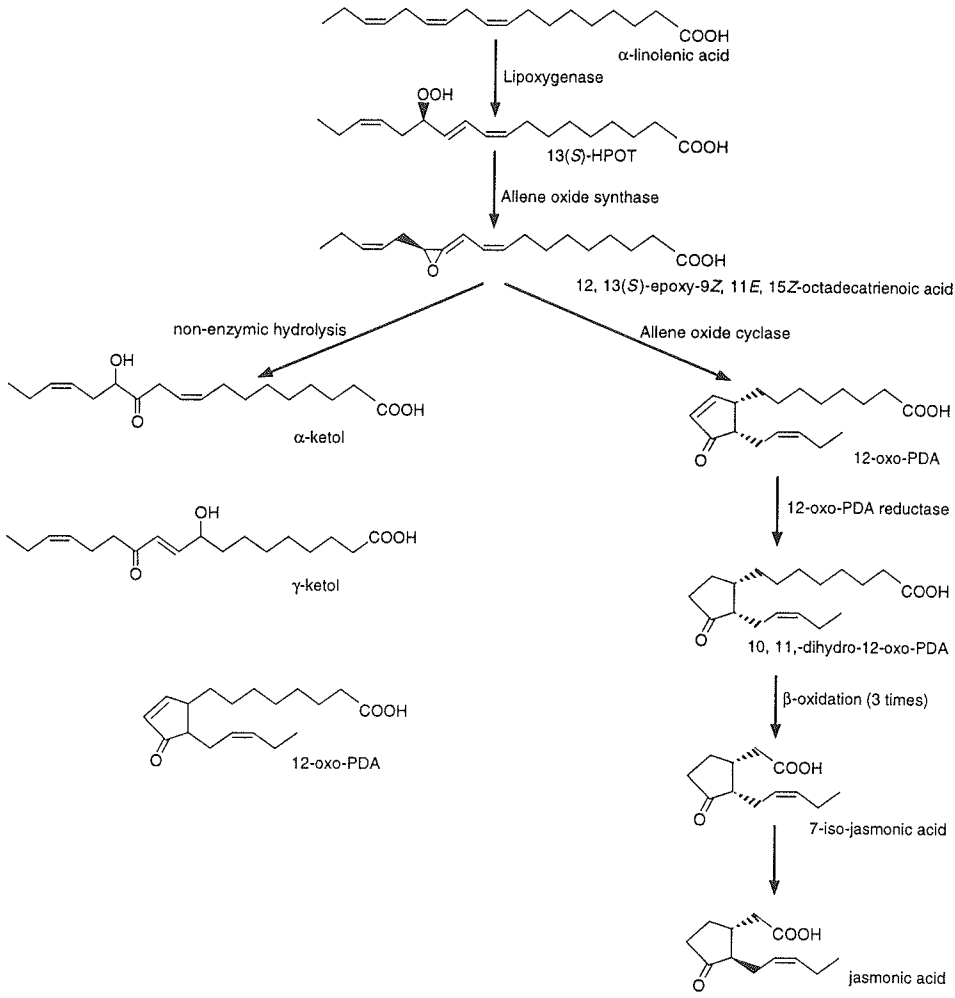

Figure 4. Production of lipoxygenase metabolites via the allene oxide synthase pathway (adapted from Van Aarle, 1993)

In the peroxygenase pathway unsaturated fatty acids are oxygenated to epoxy- or hydroxy derivatives. In this pathway fatty acid hydroperoxides, formed by the action of lipoxygenase, function as a co-substrate for the peroxygenase (epoxygenase) reaction to form epoxy acids. Subsequently, an epoxide hydrolase hydrates the epoxides into hydroxy acids. It has been proposed that by this pathway oleic acid-derived cutin monomers may be synthesized (Figure 5)(Blée and Schuber, 1993). Cutins are biopolymers and are the main component of the cuticle, which covers the aerial parts of plants, thereby forming a barrier against pathogens, herbicides, pesticides and dehydration.

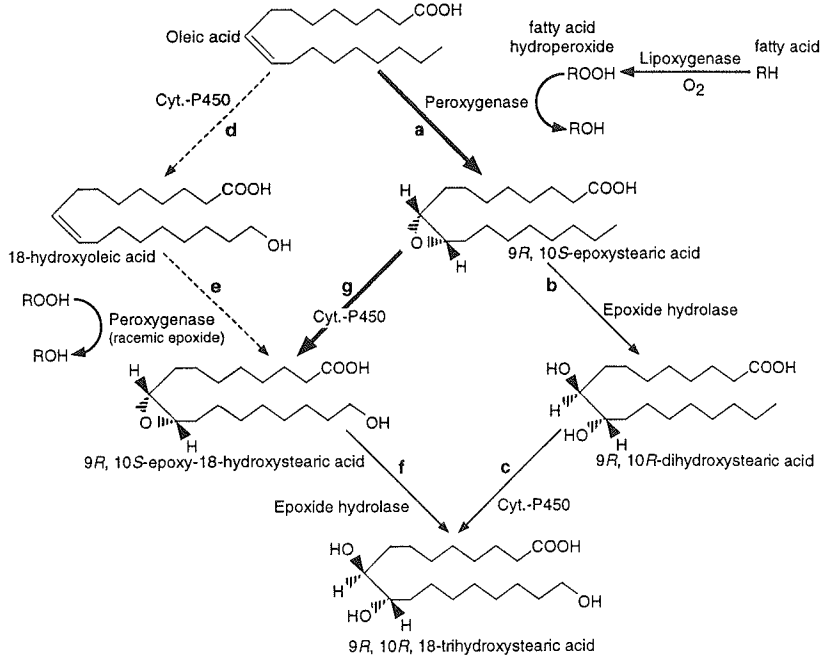

Figure 5. Scheme of the biosynthesis of C18 cutin monomers. Lipoxygenase is involved in the formation of the fatty acid hydroperoxides, the required co-substrates of the peroxygenases (adapted from Bléé and Schuber, 1993)

Recently, a new pathway has been suggested, namely the reductase pathway, in which storage acyl glycerols may be oxygenated directly by a 13-lipoxygenase into 13-hydroperoxy lipids, which subsequently are reduced by a specific reductase forming 13-hydroxy lipids (Feussner *et al.*, 1997). The hydroxy lipids are then cleaved by a lipid hydroxide-specific lipase to form hydroxy fatty acids, which may form the substrate for ß-oxidation during germination (Figure 6). In the reductase pathway the C12-C13 *cis* double bond is changed into a *trans* C11-C12 double bond. This may be an advantage for plants since the dehydratase pathway (Gerhardt and Kleiter, 1995) is by-passed, which normally is required for the degradation of linoleic acid.

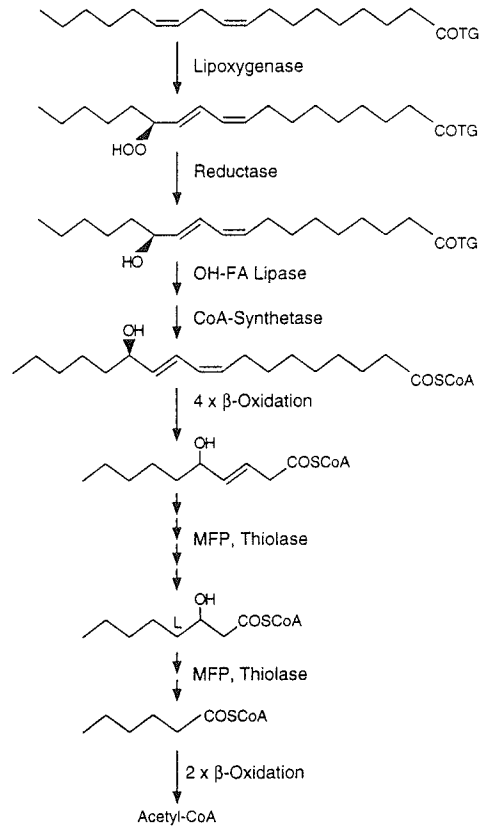

Figure 6. The reductase pathway: the lipoxygenase-dependent degradation of polyunsaturated fatty acids in plants (adapted from Feussner *et al.*, 1997)

In addition to the functions described above, lipoxygenases have been suggested to play a role in other metabolic processes, which are not so well understood. For instance, lipoxygenase has been suggested to play a role in the biosynthesis of abscisic acid (Creelman *et al.*, 1992), in the formation of divinyl ethers (Galliard and Chan, 1980), while also a role in plant growth and development (Siedow, 1991), in development of germination capacity (Hildebrand *et al.*, 1991) and in nitrogen-partitioning (Kato *et al.*, 1993) have been suggested .

In summary, the involvement of plant lipoxygenases in the synthesis of several compounds of physiological importance, such as volatile flavors and signalling molecules, is beyond doubt and points towards a role in plant responses to stress, pathogen attack and wounding. Lipoxygenases may play a role in several other metabolic processes as well, although this needs further investigation.

OUTLINE OF THIS THESIS

Cereal seed crops represent a large proportion of the diet of man and animals. Worldwide, barley ranks fourth in total production and is mainly used for making beer and feeding live stock (Simpson, 1990). Barley may contain up to 4.4 % of its total dry weight as lipid when measured as total fatty acids (Anness, 1984). About one-third of the lipid in barley is present in the germ, of which the scutellum and coleoptile contain up to 26% and 30%, respectively, of lipid on dry weight basis (Morrison, 1978). The aleurone of cereal seeds is rich in lipid as well (Galliard and Barnes, 1980). About 70% of the fatty acids of barley are present as triacylglycerols, while about 4% of the fatty acids are found as free fatty acids (Anness, 1984). Germination results in a decrease of down to 30% of the lipid content, mainly due to the hydrolysis of triglycerides and subsequent metabolism of released fatty acids (Anness, 1984).

The objective of the work described in this thesis was to investigate the expression and function of two metabolic pathways which are possibly involved in the oxidation of fatty acids during germination of barley, namely the ß-oxidation

and the lipoxygenase pathways.

In the introduction, it has been described that oxidation of fatty acids by ß-oxidation generally leads to the formation of acetyl-CoA. However, oxidation of fatty acids by lipoxygenase may lead to a broad spectrum of reaction products. These reaction products may play important physiological roles for the plant. Since several of these products have a characteristic taste and/or smell (both desirable and non-desirable), more knowledge about the expression of lipoxygenases in barley will therefore be of interest for the food industry.

Chapter 2 describes the expression of enzymes of the ß-oxidation pathway in the embryo and aleurone layer of germinating barley seeds. Organelle separation of homogenates from scutellum showed that ß-oxidation was located solely in microbodies. Enzyme activities of the glyoxylate cycle could be detected in the aleurone layer, but not in the embryo, suggesting that in this tissue ß-oxidation has a physiological function different from that in the embryo.

In **Chapter 3**, the purification and characterization of catalase from young barley leaves have been described. Catalase, the marker enzyme of microbodies, degrades hydrogen peroxide, which is produced during ß-oxidation and photorespiration. Four cell-lines were generated producing catalase-specific monoclonal antibodies, which showed differential recognition of catalase subunits in barley aleurone cells and leaves. These antibodies can be used to study the physiological significance of different catalase forms.

In **Chapter 4**, the function and regulation of expression of two different catalytic forms of catalase, namely a form showing enhanced peroxidative activity and a form showing normal peroxidative activity have been described for leaves of barley seedlings. Using the catalase-specific monoclonal antibodies, it was shown that the expression of two catalase subunits is regulated differentially by light, and that expression levels are correlated with the ratio of catalatic to peroxidative activity of catalase. A higher germination index of a catalase-deficient mutant of barley suggests a relation between the level of catalase activity at the onset of germination and the rate of germination.

The expression of lipoxygenase isoenzymes during germination in barley embryos has been described in **Chapter 5**. A detailed study has been performed of the spatial and temporal expression of lipoxygenases 1 (LOX-1) and 2 (LOX-2) during germination at the levels of activity, protein and mRNA. Differential

expression of LOX-1 and LOX-2 in barley embryos during germination suggests that these isoenzymes may have different physiological functions during germination.

In order to test this hypothesis, the substrate and product specificities of LOX-1 and LOX-2 were studied (**Chapter 6**). Kinetic data showed that LOX-2 has a higher affinity for esterified lipids than does LOX-1. Product analyses suggested that during the onset of germination LOX-2 is involved in the oxygenation of esterified polyunsaturated acids. The degradation product, 13-hydroxy fatty acid, may serve as a substrate for ß-oxidation. The results of the work described in this thesis are summarized and discussed in **Chapter 7**.

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Chapter 2

ß-Oxidation of fatty acids is linked to the glyoxylate cycle in the aleurone but not in the embryo of germinating barley

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ß-Oxidation of fatty acids is linked to the glyoxylate cycle in the aleurone but not in the embryo of germinating barley

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ABSTRACT

An assay for the measurement of overall β-oxidation was optimized for use with extracts from germinating barley. The most important adaptation is the addition of commercially available acyl-CoA synthetase to the incubation medium in order to reactivate the palmitate formed by the high thioesterase activity of the extracts.

The β-oxidation activity determined by the overall assay originated exclusively from microbodies, since inhibitors of the electron-transport chain and alternative respiration in mitochondria did not affect the total β-oxidation activity.

Activity patterns of β-oxidation upon germination were determined both for barley aleurone and embryo. In the aleurone, β-oxidation activity was already present in the quiescent grain and increased during germination, reaching an optimum 4 days after germination. In addition, in the embryo β-oxidation activity could be demonstrated in the quiescent grain, and it increased upon germination. Within the growing embryo, β-oxidation activity was present in leaves, roots and scutellum. The maximal activity levels reached in the various embryo parts were significantly lower than those in the aleurone.

Organelle separation by density gradient centrifugation of homogenates from scutellum suggests that, in this tissue, microbodies constitute the only subcellular compartment carrying out β-oxidation.

In the aleurone the developmental activity profiles of glyoxylate cycle enzymes closely resemble those of β-oxidation indicating that, in this tissue, β-oxidation is localized in so-called glyoxysomes. Inability to detect glyoxylate cycle enzyme activities in the embryo indicates different metabolic functions of the β-oxidation

route in aleurone and embryo.

Key words: barley; germination; β-oxidation; glyoxylate cycle; microbody; aleurone

INTRODUCTION

In higher plants β-oxidation plays an important role in the degradation of fatty acids. Induction of this metabolic pathway during germination was reported for oil storing tissues of oleaginous seeds as well as for non-fatty tissues from several plant species [1]. Comparison showed that enzyme activities of β-oxidation in fatty tissues were 10 to 100 times higher than in non-fatty tissues [1]. Another difference between β-oxidation in fatty and non-fatty tissues concerns its physiological function. In fatty seedling tissues, where β-oxidation is located in glyoxysomes, it is coupled to the glyoxylate cycle and gluconeogenesis, enabling conversion of lipid reserves into sugars [2].

The function of fatty acid β-oxidation in non-fatty tissues lacking an active glyoxylate cycle is not known at all. It has been suggested that β-oxidation may be involved in energy generation, turnover of membrane lipid or protein [1] and chain shortening of fatty acids to mid-chain length [3]. The prevailing idea is that in non-fatty tissues, β-oxidation is solely located in microbodies of the peroxisome type. However, recently several reports appeared suggesting a dual location of fatty acid β-oxidation, both in microbodies and mitochondria (e.g. [3]). Further investigations concerning the subcellular location of β-oxidation in plant tissues with unknown physiological function of fatty acid degradation seem appropriate.

Most cereal grains contain a relatively low amount of lipid, which is mainly stored in the aleurone layer and in the germ [4]. The lipid content in these two parts of the grain can be as high as 30% and 10% on a dry weight basis in the case of wheat germ and aleurone, respectively [5]. In this respect, the aleurone layer and the germ of cereal grains can be considered as oil storing tissues. Activity of β-oxidation has been demonstrated in wheat aleurone [6], maize scutellum [7],

seedling roots [8] and in rice coleoptiles [9]. Although the physiological function of this pathway in germinating cereal grains has not been elucidated, it is often assumed that the major function of fatty acid β-oxidation is the generation of energy for the aleurone and the growing seedling during the first stages of germination [10]. However, the presence of glyoxylate cycle enzymes in wheat and barley aleurone [6,11] and maize scutellum [7] suggests that this does not apply to all the lipid-rich tissues in germinating cereals. To investigate this further, we have studied in detail the temporal and spatial expression of enzymes of β-oxidation and the glyoxylate cycle in germinating barley. The implications for the possible physiological role of lipid degradation via β-oxidation are discussed.

MATERIALS AND METHODS

Plant materials. Barley grains (*Hordeum vulgare* L., cv. Triumph, harvest 1988) were germinated between 2 x 3 layers of moist filter paper in a plastic plant propagator (33 x 22 cm) at 25°C in the dark for 6 days. Harvested barley grains were daily dissected to obtain aleurone layers and embryos. From 2 days after germination, embryos were divided into scutella, roots and etiolated leaves (henceforth just named "leaves"). Fractionation of organelles by density gradient centrifugation was carried out using scutella isolated from seedlings germinated for 3 days.

Preparation of crude extracts. All steps were performed at 0-4°C. Crude extracts of aleurone layers and (parts of) embryos were prepared by homogenizing 10 grain parts in 1.0 ml 30 mM potassium phosphate buffer (pH=7.0), containing 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and polyvinylpolypyrrolidone (50 μg/mg tissue). After careful homogenization using an Ultra-turrax (five pulses of 24,000 rev./min of 30 s each with 30 s intervals), the resulting suspension was centrifuged for 5 min at 3,400 g. The pellet was resuspended in 1.0 ml of the same buffer without polyvinylpolypyrrolidone and homogenized during two additional pulses of 30 s. After centrifugation, for 5 min at 3,400 g, the two supernatants were combined and centrifuged for 20 min at

15,800 x g. The resulting supernatant was passed through a Sephadex G-25 column using 30 mM potassium phosphate buffer (pH=7.0) as the eluent.

Preparation of [1-^{14}C]linoleoyl-CoA. [1-^{14}C]Linoleoyl-CoA was synthesized during 20 min at 25°C in a reaction mixture containing 150 mM Tris-HCl (pH=8.5), 0.2 mM [1-^{14}C]linoleate, 2 mM CoA, 10 mM ATP and 1.5 U/ml acyl-CoA synthetase. Conversion of [1-^{14}C]linoleate into its CoA-ester was nearly complete.

Enzyme assays. Overall β-oxidation activity was measured by determining the amount of radioactively labeled [1-^{14}C]acetyl-CoA formed from radioactively labelled [1-^{14}C]palmitoyl-CoA by a modification of the method of Wanders *et al.* [12].

In this slightly modified assay, up to 75 μl extract was incubated for 10 min at 25°C in a shaking waterbath in a medium composed of 175 mM Tris-HCl (pH=8.5), 10 mM $MgCl_2$, 10 mM ATP, 50 μM FAD, 1 mM Coenzyme A, 1 mM NAD^+, 1.5 U/ml acyl-CoA synthetase (isolated from *Pseudomonas fragi*, supplied by Boehringer Mannheim) and 125 μM [1-^{14}C]palmitoyl-CoA (2.7 mCi/mmol) in a total volume of 200 μl. The reaction was stopped by mixing in a glass tube 150 μl from the incubation mixture with 1.1 ml H_2O and 3.25 ml MCH (methanol/chloroform/heptane 1.41:1.25:1.00 v/v). After shaking vigorously for 2 x 20 s, the samples were centrifuged for 5 min at 400 x g. Radioactivity was determined in 1.0 ml from the lower, organic phase, containing radioactively labeled palmitate (formed by the action of acyl-CoA thioesterase (E.C. 3.1.22)). 200 μl 2 N NaOH was added to 1.5 ml of the top, aqueous phase, containing palmitoyl-CoA, acetyl-CoA and acetate. After incubation at 50°C for 15 min to hydrolyze all CoA-esters, 40 μl concentrated H_2SO_4 was added. Subsequently, 1.5 ml of this mixture was extracted with an equal volume of water-saturated MCH as described above. Radioactivity was measured in 1.0 ml from the lower, organic phase containing [1-^{14}C]palmitic acid (representing substrate still present after incubation) and in 1.0 ml from the upper, aqueous phase containing [1-^{14}C]acetic acid. After the addition of 4.0 ml lumasafe to 1.0 ml sample, radioactivity was measured in a LKB (1209 RackBeta) liquid scintillation counter .

Acyl-CoA oxidase activity (EC 1.3.99.3) was measured essentially as described

by Gerhardt [13]. The reduction of NAD^+ in a coupled reaction with enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase (HDH) at 340 nm (ϵ= 6.2 $mmol^{-1}.l.\ cm^{-1}$) was followed. The reaction mixture contained 175 mM Tris-HCl (pH=9.5), 50 μM palmitoyl-CoA, 50 μM FAD, 3 μg/ml enoyl-CoA hydratase, 750 μM NAD^+ and 20 μg/ml HDH.

Enoyl-CoA hydratase activity (EC 4.2.1.17) was determined by a modification of the method of Overath and Raufuss [14]. The absorbance increase at 340 nm, which results from NAD^+ reduction in the coupled reaction with HDH was followed in a reaction mixture consisting of 175 mM Tris-HCl (pH=9.5), 25 μM palmitoyl-CoA, 1.0 μg/ml acyl-CoA oxidase, 20 μg/ml HDH and 750 μM NAD^+.

3-hydroxyacyl-CoA dehydrogenase activity (EC 1.1.1.35) was assayed as described for enoyl-CoA hydratase, except that HDH was omitted from the reaction mixture.

Thiolase activity (EC 2.3.1.9) was determined essentially as described by Lynen and Ochoa [15], by following the decrease in absorbance at 303 nm of the Mg-acetoacetyl-CoA complex (ϵ= 21.4 $mmol^{-1}.l.cm^{-1}$). The reaction mixture contained 100 mM Tris-HCl (pH=8.3), 25 mM $MgCl_2$, 20 μM acetoacetyl-CoA and 50 μM CoA.

Isocitrate lyase (EC 4.1.3.1) and malate synthase activity (EC 4.1.3.2) were assayed according to the method of Dixon and Kornberg [16] and catalase (EC 1.11.1.6) by the spectrophotometric method of Lück [17]. Cytochrome c oxidase (EC 1.9.3.1.) was assayed as described by Douma *et al.* [18], fumarase (EC 4.2.1.2.) according to Cooper and Beevers [19] and acyl-CoA dehydrogenase (EC 1.3.99.3) as described by Lehman *et al.* [20].

Enzyme activities are expressed per 10 aleurone layers or 10 embryos. Similar patterns were obtained when the activity was expressed per mg protein, indicating that the patterns found are not caused by differences in extraction efficiency. Protein concentrations were determined by the BCA protein assay, using BSA as a standard [21].

Preparation of organelle fractions. All steps were carried out at 0-4°C. The plant material (500 scutella) was ground in 40 ml homogenization buffer with a mortar and pestle. Homogenization buffer consisted of 50 mM potassium phosphate (pH=7.5), containing 0.5 M sucrose, 1 mM EDTA and 1 mM PMSF. After

filtration over a nylon cloth the filtrate was centrifuged at 500 x g for 10 min in a Sorvall RC 5B-GSA rotor. Subsequently, the supernatant was loaded on a cushion consisting of 3.0 ml 60% (w/w) sucrose in 50 mM potassium phosphate buffer (pH=7.5) followed by centrifugation at 10,000 x g for 30 min in a Kontron T-2070 ultracentrifuge using a TST 28.38 rotor. The pellet floating on the cushion which contained concentrated organelles was gently collected and layered on top of a discontinuous sucrose gradient composed of 50 mM potassium phosphate buffer (pH=7.5) with the following sucrose concentrations (w/w): 4.0 ml 60%, 5.0 ml 56%, 5.0 ml 52%, 5.0 ml 46%, 5.0 ml 42% and 9.0 ml 36%. After centrifugation for 3 h at 80,000 x g (same ultracentrifuge and rotor as described above), fractions (1.0 ml) were collected and assayed for enzyme activities.

Electron microscopy. The mitochondrial and microbody fractions, obtained by density gradient centrifugation, were fixed with an equal volume of 0.2 M cacodylate buffer (pH=7.2) containing 6% glutaraldehyde for 30 min at 0°C. After centrifugation (20 min at 15,800 x g in an Eppendorf centrifuge), the samples were rinsed in 0.1 M cacodylate-buffer (pH=7.2) followed by post-fixation in the same buffer containing 1% osmium tetroxide. The samples were dehydrated in a graded ethanol series. After impregnation with propylene oxide, the specimen passed a series of 5 propylene oxide/Epon 21045 mixtures with an increasing Epon concentration. Ultrathin sections were stained with saturated aqueous uranyl acetate and lead citrate solutions for 5 min each, and were examined with a Philips 300 electron microscope operating at 60 kV.

RESULTS

Assay for the measurement of overall fatty acid β-oxidation

In order to determine activity patterns of β-oxidation in the aleurone and the germ of germinating barley, we used a procedure based on the method of Wanders *et al.* [12] for extracts of human liver. This method allows the simultaneous measurement of acetyl-CoA formed from palmitoyl-CoA via β-oxidation and palmitate produced due to the action of thioesterases. With this slightly modified

assay, overall β-oxidation activity could be detected in extracts of barley aleurone of 4 days germinated grains, using a basic incubation medium, which apart from the substrate (palmitoyl-CoA), contained NAD^+, FAD, $MgCl_2$, ATP and Coenzyme A. Under these circumstances the amount of palmitate formed was about 2-3 times higher than the amount of acetyl-CoA produced by β-oxidation. The ratio of palmitate to acetyl-CoA formation was even higher in the abscence of ATP, when the reactivation of palmitate to palmitoyl-CoA by acyl-CoA synthetase present in the extracts is not possible (data not shown). These results indicate the presence in the aleurone extracts of high thioesterase activity consuming the substrate for β-oxidation. This may strongly interfere with measurements of the activity of the overall β-oxidation. We therefore attempted to reactivate the palmitate produced to palmitoyl-CoA by the addition of acyl-CoA synthetase (isolated from *Pseudomonas fragi*) to the incubation medium.

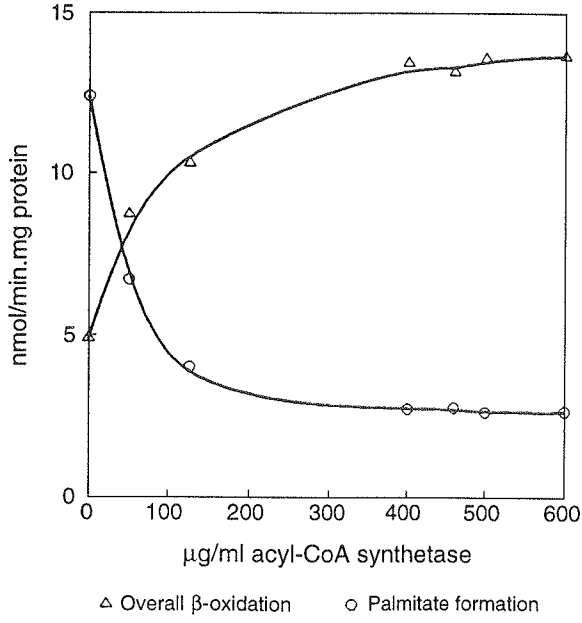

Figure 1. Effect of increasing concentrations of acyl-CoA synthetase on the rate of overall β-oxidation and palmitate formation in barley aleurone extracts using [1-^{14}C]palmitoyl-CoA as a substrate.

The results depicted in Fig. 1 demonstrate that the addition of this enzyme strongly reduces the amount of palmitate formed. It also results in an increase of the activity of β-oxidation, probably due to relief of substrate limitation. The acyl-CoA synthetase preparation alone did not contain activities of individual enzymes of the β-oxidation reaction. In subsequent experiments 300 μg/ml (1.5 U/ml) acyl-CoA synthetase was routinely added to the incubation medium .

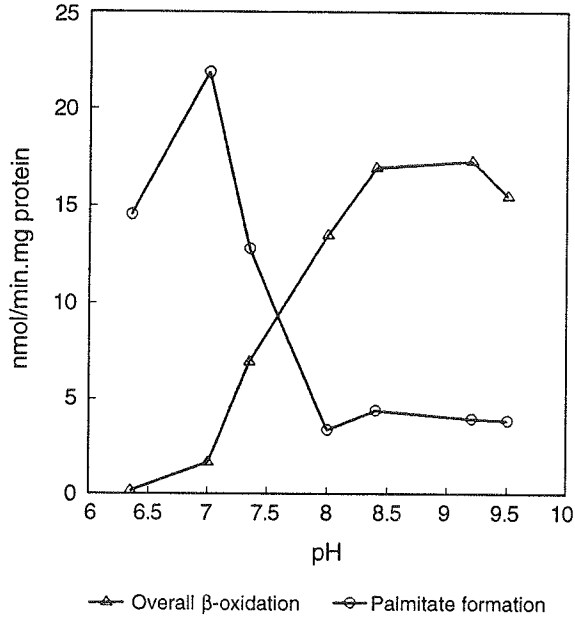

Figure 2. pH-dependency of the rate of overall β-oxidation and palmitate formation in barley aleurone extracts using [1-^{14}C]palmitoyl-CoA as a substrate

In addition we investigated the influence of the pH on the overall β-oxidation activity and palmitate formation in barley aleurone extracts. Fig. 2 shows that overall β-oxidation activity has a broad pH-optimum between pH 8.0 and 9.5, which is comparable to results obtained for human liver [12]. At these pH values the rate of palmitate formation is low and has an optimum around pH 7.0. Based

on these results, we decided to carry out the assay at pH 8.5.

Furthermore, the effect of substrate concentration on overall β-oxidation activity was studied. It appeared that palmitoyl-CoA oxidation reaches a plateau at approximately 100 μM of substrate (data not shown). From these results, we calculated an apparent K_m value of approximately 35 μM for overall β-oxidation activity in barley aleurone for palmitoyl-CoA, which is comparable to results obtained for human liver [12]. In subsequent experiments a substrate concentration of 125 μM was chosen.

The assay for overall β-oxidation activity only determines the activity of microbody β-oxidation. Mitochondrial β-oxidation is dependent on the presence of an active electron transport chain, which is not a prerequisite for microbody β-oxidation. Since all measurements were carried out in barley extracts which were not osmotically stabilized, it is unlikely that intact mitochondria were still present.

Table 1. Determination of the rate limiting step in fatty acid β-oxidation in barley, by addition of commercially available enzymes to the reaction mixture.

Addition	Activity in aleurone (%)	Activity in scutellum (%)
None	100	100
Acyl-CoA oxidase (10 μg/ml)	736	1656
Enoyl-CoA hydratase (10 μg/ml)	111	103
Hydroxyacyl-CoA dehydrogenase (100 μg/ml)	152	103
SHAM (2 mM)	114	98
KCN (2 mM)	100	103
KCN + SHAM (both 2 mM)	91	121

Overall β-oxidation activity was expressed as a percentage of the activity obtained without additions. The influence of KCN, salicylhydroxamic acid (SHAM) and KCN + SHAM on overall β-oxidation activity was also determined.

This was further corroborated by experiments in which KCN (inhibitor of electron transport chain) or salicylhydroxamic acid (inhibitor of alternative oxidation) or a combination of both inhibitors were added to the incubation mixture. These inhibitors did not significantly affect the β-oxidation activity (Table 1).

β-Oxidation activity in germinating barley

Using the assay for overall β-oxidation described above, activity patterns in aleurone and embryo during germination were established. In the aleurone, overall β-oxidation activity is already present in the quiescent grain. During the first day of germination, the activity decreased, but subsequently increased by approximately five-fold reaching an optimum at day 4 (Fig. 3).

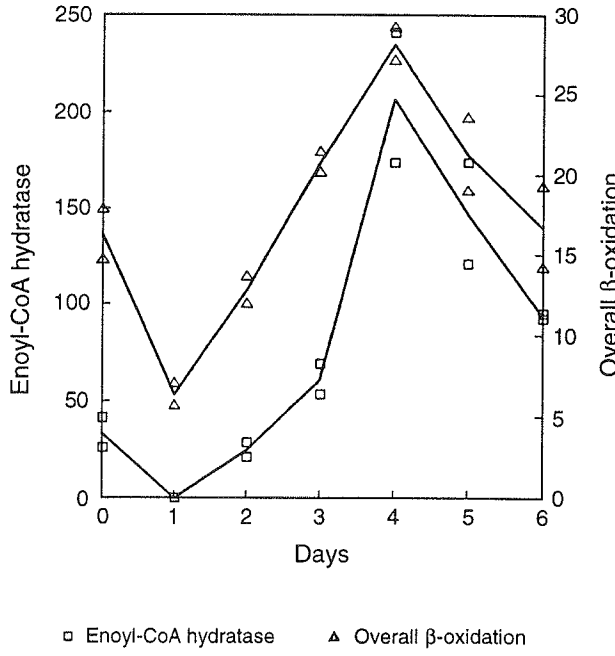

Figure 3. Time course of enoyl-CoA hydratase and overall β-oxidation activities in the aleurone of germinating barley. Activities are expressed as nmol/min/10 aleurone layers.

In addition, in the embryo β-oxidation activity was already present in the dry grain and it increased slightly during the first day (Fig. 4A). From 2 days after germination, dissection of the embryo into scutella, leaves and roots has been performed. In the scutellum, highest activity values were obtained 2-3 days after germination, followed by a gradual activity decrease (Fig. 4A). Activity of β-oxidation in roots was initially low, but increased during germination, reaching optimal values after 4 days of germination. Activities in the leaves remained at a relatively constant level. The maximal activity of β-oxidation in the different parts of the embryo was significantly lower than that in the aleurone.

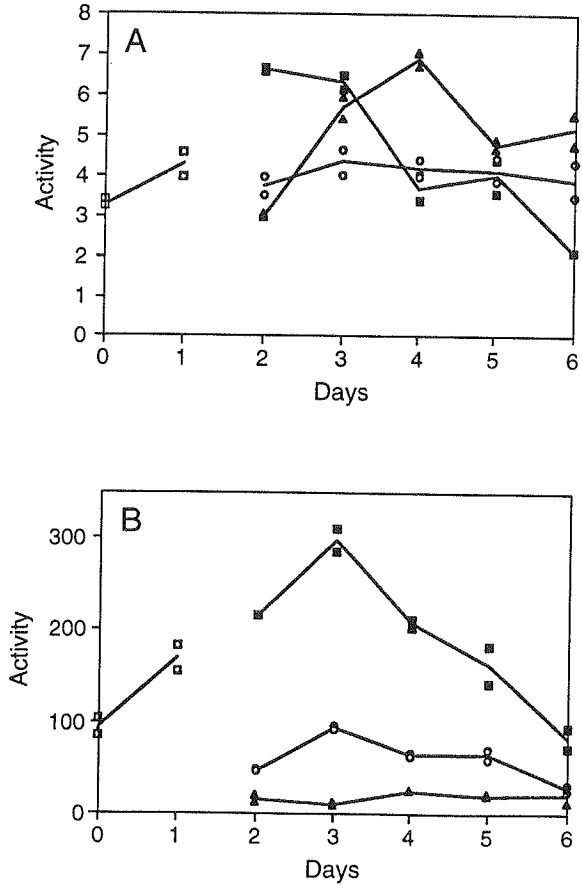

Figure 4. Time course of overall β-oxidation (A) and enoyl-CoA hydratase activity (B) in the embryo (□), scutellum (■), leaf (○) and root (▲) of barley upon germination. Activities are expressed as nmol/min/10 (parts of) embryos.

Apart from overall β-oxidation activity, activities of individual β-oxidation enzymes, namely acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and thiolase were detectable in scutella and aleurone at the time when optimal β-oxidation activity was obtained (data not shown). For the enzyme with the highest activity, namely hydratase, activity patterns during germination were established. Comparison with those of overall β-oxidation showed remarkable similarity in aleurone, scutellum and leaf (Figs. 3 and 4B). In the roots the hydratase activity pattern differed from that of overall β-oxidation. In this part of the embryo activity was low and remained constant with time (Fig. 4B).

In order to determine which enzyme catalyzes the rate-limiting step *in vitro*, commercially available acyl-CoA oxidase, enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase was added to the reaction medium in the overall β-oxidation assay. Purified thiolase was not available. Measurements in extracts of aleurone and scutella showed that overall β-oxidation activity only increased significantly after addition of acyl-CoA oxidase, indicating that probably this enzyme catalyses the rate-limiting step *in vitro* (Table 1). Of course it has to be kept in mind that the *in vitro* situation does not necessarily represent the *in vivo* situation. Thiolase was suggested to be the rate-limiting step in cucumber extracts *in vitro* [22].

Linoleoyl-CoA, the most abundant fatty acid in quiescent barley grains [4], can also be used as a substrate in the overall β-oxidation assay. However, activities detected in the aleurone were 2 times lower, while in the scutellum activities were comparable to those found using palmitoyl-CoA as a substrate.

To investigate the possible fate of acetyl-CoA produced by β-oxidation, we assayed for activities of marker enzymes of the glyoxylate cycle, i.e. malate synthase and isocitrate lyase. In the aleurone, the activity patterns of these enzymes closely resemble those of β-oxidation, indicating that in this tissue β-oxidation is localized in glyoxysomes (Fig. 5). Therefore, in barley aleurone, lipid reserves may be used for the production of sugars. In the embryo, enzyme activities of the glyoxylate cycle could not be detected. This is not due to the action of an inhibitor, as described for isocitrate lyase from ripening cucumber seeds [23] since crude extracts of scutellum did not inhibit glyoxysomal enzyme activities in aleurone extracts (data not shown). Consequently in the embryo β-oxidation probably has another physiological function than in the aleurone.

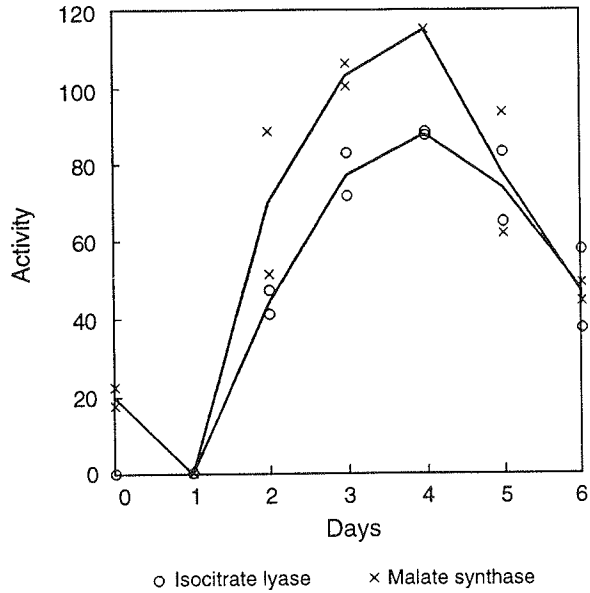

Figure 5. Time course of isocitrate lyase and malate synthase activities in the aleurone of germinating barley. Activities are expressed as nmol/min/10 aleurone layers.

Subcellular location of β-oxidation in barley scutellum

More insight into the role of β-oxidation in the embryo may be obtained by elucidating its subcellular location. Although in most higher plants β-oxidation is located in microbodies, recently a dual location of β-oxidation in microbodies and mitochondria has been described for pea [24], *Arum* and oilseed rape [25] and avocado [3].

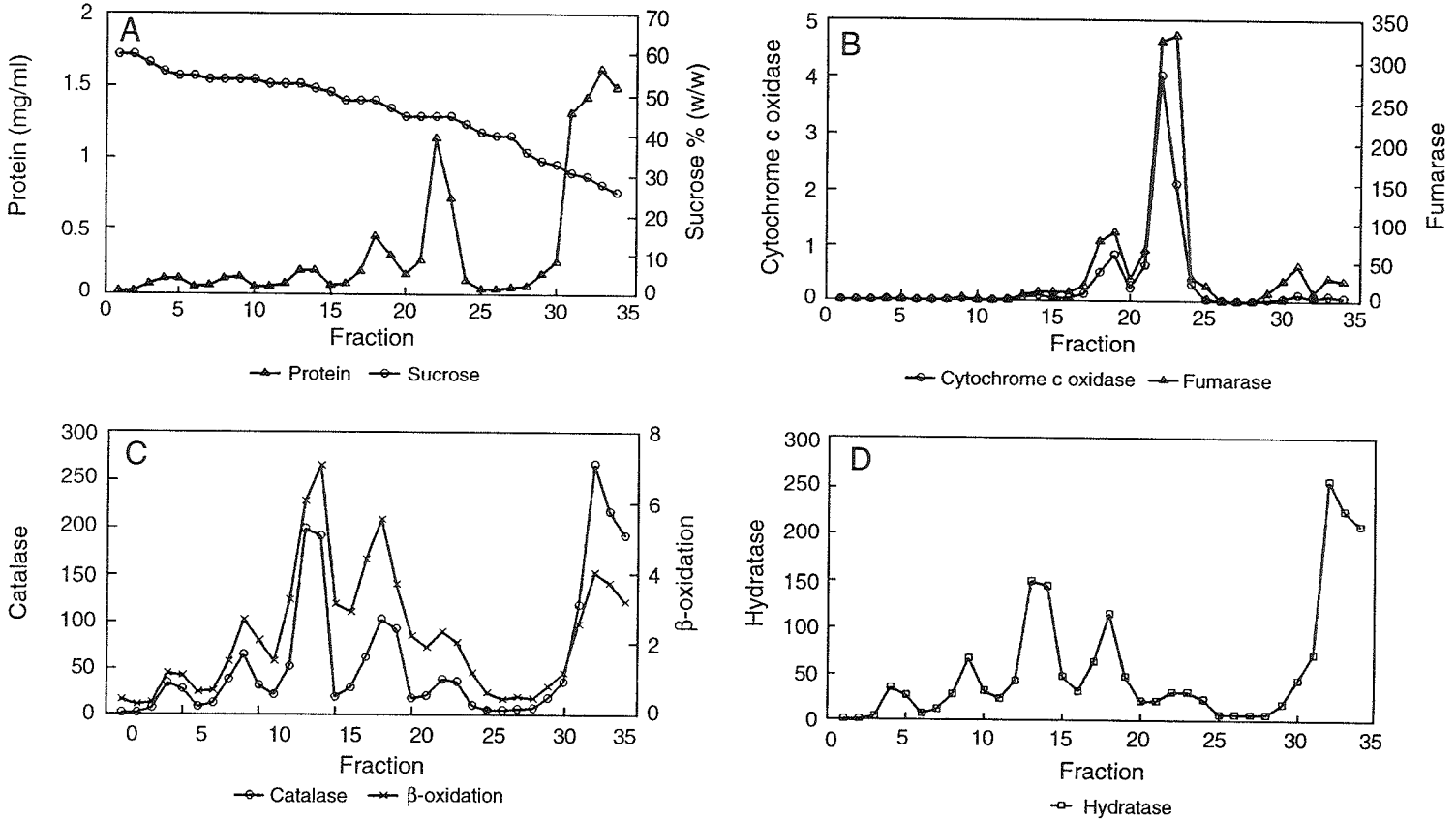

Figure 6. Distribution of enzyme activities after density gradient centrifugation of a resuspended pellet enriched in microbodies. This pellet was obtained after differential centrifugation of homogenates from barley scutella. Activities are expressed as nmol/min/ml, except for cytochrome c oxidase and catalase which are in μmol/min/ml.

We investigated the subcellular location of β-oxidation in barley scutellum. After homogenization of the scutella and a 500 x g centrifugation step to remove debris and large organelles, the 10,000 x g pellet was loaded on a discontinuous sucrose gradient. After centrifugation, 3 distinct bands could be observed at approximately 45% (1.20 $g.cm^{-3}$), 48% (1.22 $g.cm^{-3}$) and 53% (1.24 $g.cm^{-3}$) sucrose (Fig. 6A). The distribution of the mitochondrial marker enzymes cytochrome c oxidase and fumarase in the gradient (Fig. 6B), as well as electron microscopy (Fig. 7A) indicated that the band at 45% sucrose mainly consists of mitochondria. Some contamination of this fraction with microbodies was observed, as can be concluded from the small activity peak of the microbody marker enzyme, catalase, in this fraction. Microbodies were enriched especially in the band at 53% sucrose. This band contained the highest activity of catalase (Fig. 6C). In addition, the relatively abundant presence of intact microbodies could easily be demonstrated microscopically (Fig. 7B). In this fraction, membrane vesicles were also observed, probably as a result of rupture of the very fragile microbodies, which is in agreement with the high catalase activity detected at the top of the gradient. The microbody peak fraction was not completely pure and contained some mitochondria and plastids. The nature of the protein band at 48% sucrose has not been investigated in detail. Electron microscopy suggests that it mainly consists of plastids (data not shown).

Table 2. Activity of β-oxidation enzymes and marker enzymes of microbodies (catalase) and mitochondria (fumarase and cytochrome c oxidase) in microbody and mitochondrial fraction of barley scutellum

Enzyme	Activity (nmol min^{-1} $fraction^{-1}$)		
	Microbody fraction (I)	Mitochondrial fraction (II)	Ratio (I/II)
Catalase	197900	36800	5.4
Hydratase	151	32	4.7
Overall ß-oxidation	6.0	2.3	2.6
Cytochrome c oxidase	66	4028	1.6×10^{-2}
Fumarase	7.7	323	2.4×10^{-2}

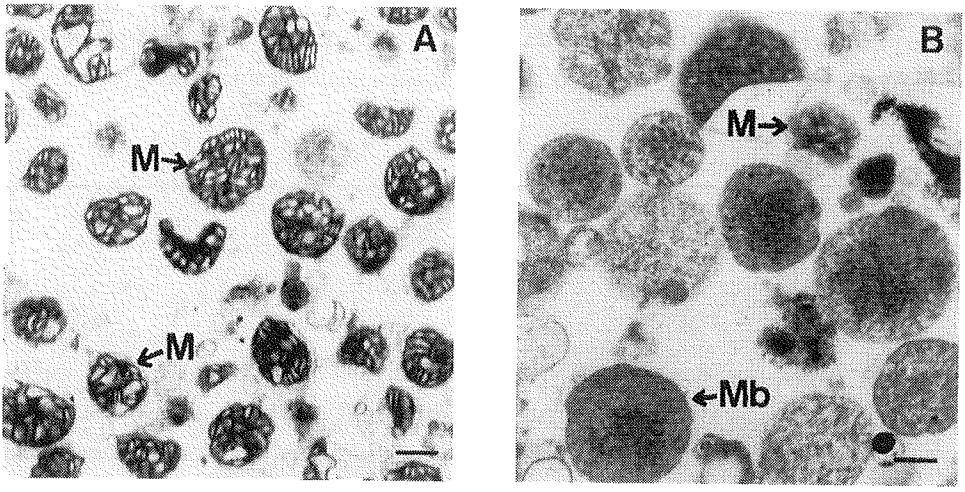

Figure 7. Electron micrographs of the mitochondrial (A) and microbody fraction (B) of barley scutellum isolated after density gradient centrifugation. Abbreviations: M, mitochondrion; Mb, microbody. The bar represents 0.4 μm.

In order to investigate the subcellular location of β-oxidation the gradient fractions were further assayed for activity of β-oxidation enzymes. Fig. 6C shows that the activity pattern of overall β-oxidation (with which only microbody β-oxidation is determined) parallelled that of catalase. The pattern of hydratase activity (Fig. 6D) was similar to that of catalase and overall β-oxidation.

Furthermore, the ratio of activity of this enzyme in the microbody fraction and the mitochondrial fraction, namely 4.7, was comparable to that of catalase, and even higher than that for overall β-oxidation (Table 2). For both peak fractions, hydratase activity with octanoyl-CoA was approximately 5-fold lower than with palmitoyl-CoA. These results suggests that there is no, or only a minor, mitochondrial contribution in the hydratase activity.

The possibility of mitochondrial β-oxidation was further investigated by assaying acyl-CoA dehydrogenase, the first enzyme of mitochondrial β-oxidation. Whereas high enzyme activities were detected in controls using extracts of pig liver, no activity of this enzyme was found in the mitochondrial peak fraction. This is in agreement with the inability to detect acyl-CoA dehydrogenase in non-fatty plant

tissues (summarized by [1]).

As is the case in extracts of whole embryos, no activities of enzymes of the glyoxylate cycle could be detected in the microbody peak fraction, indicating that in the embryo β-oxidation is located in microbodies of the peroxisome type.

DISCUSSION

In this paper, we describe patterns of spatial and temporal expression of β-oxidation of fatty acids in the course of germination of barley. The results were obtained using an overall β-oxidation assay, which was modified for use in extracts containing a relatively high thioesterase activity.

Apart from the enzymes necessary for the conversion of palmitoyl-CoA into acetyl-CoA most probably also acyl-CoA synthetase, which catalyzes the activation of palmitate to palmitoyl-CoA, was present in the extract. This was concluded from the observation that during measurement of overall β-oxidation in the presence of ATP, which is essential for acyl-CoA synthetase activity, less palmitate is formed than in its absence. This suggests that acyl-CoA synthetase present in the extracts reactivates part of the palmitate formed by thioesterases.

In wheat aleurone β-oxidation is induced by the hormone gibberellic acid (GA_3) [6]. The fact that lipid degradation in barley aleurone layers [26] and aleurone protoplasts [27] is stimulated by the addition of GA_3 suggests that this could be the case for barley as well.

From our results (Fig.6, Table 1 and 2) we have no indications that β-oxidation in barley is, beside in microbodies, also located in mitochondria, as was described for pea [24], *Arum* and oilseed rape [25]) and avocado [3]. By subcellular fractionation of scutellum homogenates, no experimental evidence for the existence of mitochondrial β-oxidation could be obtained. In addition, it was not possible to detect acyl-CoA dehydrogenase in the mitochondrial peak fraction.

The presence of an active glyoxylate cycle in barley aleurone indicates that, in this tissue, the β-oxidation route for the degradation of fatty acids is localized in glyoxysomes as was already described for fatty tissues from oleaginous seeds. As

in these oleaginous seeds, the end product of lipid degradation may be sugars. This assumption is supported by the observation that isolated barley aleurone layers are capable of synthesizing and secreting sucrose [28]. Alternatively lipid conversion may lead to the production of malate. Malate was shown to be secreted by isolated aleurone layers [29] and may be responsible for the acidification of the endosperm in germinating grains, resulting in an increased response of the aleurone layer to GA_3 [30].

In the embryo, activities of the glyoxylate cycle have not been detected. This suggests that in this part of the grain, as in non-fatty tissues, lipid degradation is located in peroxisomes and may lead to energy production. The acetyl-CoA produced should then be transported to mitochondria via a currently unknown mechanism. These results differ from those in maize where glyoxysomal activities were shown to be present in the scutellum [31]. Apparently not all cereals behave similarly in this respect.

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Chapter 3

Monoclonal antibodies for differential recognition of catalase subunits in barley aleurone cells

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Monoclonal antibodies for differential recognition of catalase subunits in barley aleurone cells

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ABSTRACT

For the production of monoclonal antibodies catalase was purified 2000-fold from leaves of 6-days-old barley (*Hordeum vulgare*) seedlings. The subunit and native molecular mass of the enzyme was determined to be 55 and 245 kDa respectively, indicating a tetrameric structural organization. The isoelectric point of the enzyme was judged to be 6.0. Barley catalase exhibits an activity optimum in potassium phosphate buffer between pH 6.0 and 8.0 and in Tris-HCl buffer between pH 8.0 and 9.5. The enzyme showed cross-reactivity with polyclonal antibodies against maize catalase-1 on Western blots of SDS-polyacrylamide gels.

Using this catalase preparation four stable hybridoma cell lines were obtained producing catalase-specific monoclonal antibodies which recognized both leaf and aleurone catalase subunits. Three antibody lines (39.3, 39.10, 39.14) reacted with epitopes of the single catalase subunit (55 kDa) present in leaves as well as with epitopes of the two subunits (57 and 51 kDa) in the aleurone cells. Line 39.5 reacted in Western blots with the leaf 55 kDa and the aleurone 51 kDa subunit, but not with the 57 kDa aleurone subunit.

Immunocytochemistry with a mixture of two monoclonal antibodies revealed a specific labelling of the microbody matrix both in young leaves and in aleurone and scutellum tissues. These results were confirmed by cytochemical staining of catalase activity. The antibodies will be useful in exploring the question, whether the leaf and aleurone catalase polypeptides are synthesized by transcription from one or more genes.

Key words: purification; (immuno)-cytochemistry; microbody; *Hordeum vulgare*.

Abbreviations: AT, 3-amino-1,2,4-triazole; BSA, bovine serum albumin; CAT, catalase; DAB, 3,3'-diaminobenzidine tetrahydrochloride; PBST, phosphate buffered saline containing 0.05% (v/v) Tween 20; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecyl sulphate - polyacrylamide gel electrophoresis; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

INTRODUCTION

Catalase (EC 1.11.1.6) dismutates hydrogen peroxide into oxygen and water. It is a tetrameric, four heme-containing enzyme found in higher plants a.o. in peroxisomes of leaves and glyoxysomes of lipid storing tissues. In leaf peroxisomes catalase removes hydrogen peroxide mainly generated by glycolate oxidation and in glyoxysomes that produced by acyl-CoA oxidation in the ß-oxidation of fatty acids.

In all plants so far investigated in detail, multiple forms of catalase have been detected. Thus, in maize three distinct catalase (CAT) isoenzymes are encoded by three structural genes (Bethards *et al.*, 1987; Redinbaugh *et al.*, 1988). The sequence of cDNA clones has revealed the subunits of CAT-1 to contain 492 amino acid residues (56,738 Da), those of CAT-2 483 residues (roughly 53,000 Da), while CAT-3 subunits had a sequence of 495 residues (56,661 Da). CAT-3 is found prominently in the mesophyll cells of light-and dark-grown shoots, while CAT-1 is the major isoenzyme of the developing endosperm and of the scutellum during germination. CAT-2 with its lower molecular mass subunits is present in peroxisomes of bundle sheath cells but also in scutella during germination and in the aleurone during kernel development.

Among the three isoenzymes amino acid sequence similarity is high (53-80%) for the ß-barrel domain, the subunit-subunit interaction domain and the wrapping domain, containing important residues for catalysis. The carboxy-terminal domain, forming a hydrophobic channel to the catalytic site is the least conserved (37-50%). Heterotetrameric molecules consisting of CAT-1 and CAT-2 subunits are present in the microbodies *in vivo* and CAT-3 forms heterotetramers *in vitro* with CAT-2.

In germinating pumpkin seeds (*Cucurbita* sp.) the microbodies in cotyledons contain catalases with molecular masses of 230,000 and 215,000 Da (Yamaguchi *et al.*, 1986). The higher molecular mass form is assembled from 55 kDa subunits in glyoxysomes to an enzyme with high catalytic activity. During the conversion of glyoxysomes to peroxisomes in greening cotyledons, the lower and less active molecular mass form with 59 kDa subunits gradually replaces the high molecular mass form.

The primary structure of the catalase subunit of dark-grown cotton cotyledons has been deduced from a cDNA clone (492 amino acids, 56,800 Da) by Ni *et al.* (1990) and that of roots from sweet potato (492 amino acids, 56,984 Da) by Sakajo *et al.* (1987). Subunit composition has been studied for 8 catalase isoforms of greening sunflower cotyledons (Eising *et al.*, 1990). The glyoxysomes contained tetramers consisting of 55 kDa subunits while the leaf-type peroxisomes contained tetramers which included higher molecular mass subunits of 59 kDa.

Since relationships between the various subunits, possible processing and posttranslational modification inside the microbodies as well as the physiological significance of the multiple enzyme forms remain to be clarified, we have produced and characterized monoclonal antibodies which can distinguish the subunits of catalase found in barley leaves and aleurone cells.

In the mesophyll of barley leaves, peroxisomes are closely associated with chloroplasts and contain catalase as evidenced by reaction product made visible in electron micrographs with diaminobenzidine (DAB) (Parker and Lea, 1983). These reaction products are absent in peroxisomes of mutant RPr 79/4 which lacks 90% of leaf catalase activity (Kendall *et al.*, 1983; Lea *et al.*, 1992). The mutant is devoid of the two leaf catalase isoenzymes detectable by starch-gel electrophoresis. Barley leaf catalase had a molecular mass of 230,000 Da (Kendall *et al.*, 1983) and the homozygous recessive mutant seedlings survive only in a high CO_2 atmosphere in which photorespiration is dispensable. Microbodies have been identified by electron microscopy in the aleurone (Gram, 1982) and scutellum cells of germinating barley grains but their enzymatic contents have not yet been analyzed.

MATERIALS AND METHODS

Plant material. Barley grains (*Hordeum vulgare* L., cv. Triumph, harvest 1988/1989) were germinated between 2 x 3 layers of moist filter paper in a plastic plant propagator (33 x 20 cm) at 25° C in the dark. After 2 d the seedlings were exposed to light (alternately 16 h light and 8 h dark). Leaves were harvested from 6-d-old plants. Aleurone layers were prepared from grains germinated for 4 d in the dark at 25° C, except for immunocytochemical studies where this tissue was isolated from grains germinated for 2 d under the same conditions.

Chemicals. Phenylmethylsulphonyl fluoride (PMSF), hypoxanthine, azaserine, p-nitrophenyl phosphate, 3-amino-1,2,4-triazole (AT), 3,3,'-diaminobenzidine tetrahydrochloride (DAB) and protein A gold particles (10 nm in diameter) were purchased from Sigma. Bovine liver catalase, lactate dehydrogenase, xanthine oxidase, citrate synthase and horse radish peroxidase were obtained from Boehringer. PBE 94, Sephacryl S-300, Mono P HR 5/20, Superose 12 HR 10/30, Polybuffer 74, homogeneous polyacrylamide gels 12.5% and isoelectric focusing gels pH 3-9 were supplied by Pharmacia.

The bicinchoninic acid protein assay reagent was purchased from Pierce, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate and alkaline phosphatase were from Promega and nitrocellulose was from Schleicher and Schuell. Complete and incomplete Freund's adjuvant were obtained from Difco, glutamine and kanamycin from Flow laboratories, bovine calf serum from Hy Clone, and Dulbecco's modified Eagle's medium, penicillin and streptomycin from Gibco. Polyclonal antibodies against maize catalase-1 were a gift from Dr. J.G. Scandalios, North Carolina State University, USA. Purified barley (1-3)(1-4)-ß-glucanase-2 and monoclonal antibodies against barley α-amylase-2 were kindly supplied by Dr. K.K. Thomsen and Dr. X. Wang respectively, Carlsberg Laboratory, Copenhagen, Denmark.

Purification of catalase. All steps were performed at 4° C. The use of ion exchange columns for the purification of catalase was avoided, since the enzyme was largely inactivated on such columns. Leaves (4.7 kg) were homogenized in a Waring blender in 5.2 l buffer A (25 mM bisTris-HCl buffer (pH 7.0), containing

1 mM $MgCl_2$ and 1 mM PMSF). The homogenate was passed through a nylon cloth and subsequently centrifuged at 10,240 x g in a Sorvall RC 5B-GSA rotor for 3 h. The supernatant was filtered through Millipore HA filters (12 μm, 5 μm, and 1.2 μm successively), diluted to an osmolality of 170 milli-osmol and loaded onto a PBE 94 chromatofocusing column (5.0 x 30 cm).

After washing the column with buffer A, it was eluted with 25 mM piperazine-HCl buffer (pH 5.0) containing 1 mM $MgCl_2$ and 1 mM PMSF at a flow rate of 450 ml h^{-1}. Peak fractions containing catalase activity were pooled and concentrated on an Amicon ultrafiltration unit, equipped with a 30 kDa cut-off filter. The concentrated enzyme solution was applied to a Sephacryl S-300 column (2.6 x 100 cm), which was equilibrated with buffer B (15 mM Tris-HCl buffer (pH 8.0), containing 1 mM $MgCl_2$ and 1 mM PMSF). Elution was performed with the same buffer at a flow rate of 15 ml h^{-1}.

Peak fractions containing catalase activity were pooled, dialysed overnight against buffer A and subjected to chromatofocusing using a Mono P HR 5/20 column. After washing the column with buffer A, it was eluted with Polybuffer 74 (pH 5.0) at a flow rate of 30 ml h^{-1}. Peak fractions were pooled and concentrated as described above. The concentrated enzyme solution was loaded onto a Superose 12 HR 10/30 column which had been equilibrated with buffer B. Elution was carried out with the same buffer at a flow rate of 6 ml h^{-1}. Fractions containing the highest catalase activity were pooled and stored at -80°C.

Enzyme assays and protein determination. The activity of catalase (EC 1.11.1.6) was assayed according to the spectrophotometric method of Lück (1963), citrate synthase (EC 4.1.3.7) as described by Srere (1969), xanthine oxidase (EC 1.1.3.22) according to the method of Bergmeyer *et al.* (1983) and lactate dehydrogenase (EC 1.1.1.27) as described by Doderer *et al.* (1992). Protein was determined by the bicinchoninic acid protein assay, using bovine serum albumin (BSA) as a standard (Smith *et al.*, 1985).

Gel electrophoresis. Crude extracts were prepared by homogenizing 10 aleurone layers or 20 mg leaves in 1 ml 50 mM potassium phosphate buffer (pH 7.0), containing 1 mM $MgCl_2$ and 1 mM PMSF. After careful homogenization at 4°C using an Ultra-turrax (five pulses of 24,000 rpm of 30 s each with 30 s intervals),

the resulting suspension was centrifuged at 4°C for 15 min at 16,000 x g in an Eppendorf centrifuge.

Native PAGE or SDS-PAGE was performed either with the Phast System (Pharmacia) according to the manufacturers' instructions or using the Midget system (LKB 2050) according to the method of Laemmli (1970). Isoelectric focusing was conducted using Phast System gels covering a pH range of 3-9.

Silver staining was performed according to the method of Heukeshoven and Dernick (1988), except that native polyacrylamide gels were subjected to a 10 min incubation step with 20% trichloroacetic acid prior to the staining procedure. Staining of gels for catalase activity with DAB was carried out using the procedure of Clare *et al.* (1984). In control experiments 16 mM AT was added during preincubation and incubation.

Western blotting. Transfer of proteins from the gels onto nitrocellulose membranes was achieved by semi-dry electroblotting essentially as described by Towbin *et al.* (1979) and Kyhse-Andersen (1984). After blotting, the nitrocellulose membranes were soaked in phosphate buffered saline containing 0.05% (v/v) Tween 20 (PBST) plus 1% (w/v) BSA for 30 min and incubated overnight at 25°C with polyclonal or monoclonal antibodies diluted in the same buffer. Subsequently, the membranes were washed with PBST and incubated for 1 h at 25°C with either goat anti-rabbit IgG or goat anti-mouse IgG conjugated to alkaline phosphatase. Finally, the bound alkaline phosphatase activity was visualized by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as the substrate. Colour development was stopped by washing the nitrocellulose membranes with distilled water.

Sedimentation velocity centrifugation. In order to estimate the molecular mass of barley catalase, the purified enzyme was centrifuged on a 11.2 ml linear sucrose gradient (3-20% w/v). Enzyme solution (0.2 ml), containing partially purified catalase (4.3 μg) and the internal markers xanthine oxidase (1 mg; 275 kDa), lactate dehydrogenase (20 μg; 140 kDa) and citrate synthase (20 μg; 87 kDa) was layered on top of the gradient. Centrifugation was carried out at 86,000 x g for 16 h at 4°C in a Kontron Ultracentrifuge T-2070 using a TST 28.38 rotor. After centrifugation, 0.25 ml fractions were collected and assayed for activities of

catalase and the different marker enzymes.

Production and characterization of monoclonal antibodies. Female BALB/c mice were immunized intraperitoneally with 80 μg of the partly purified catalase preparation in 200 μl of an emulsion consisting of equal volumes of PBS and complete Freund's adjuvant. Three weeks later a booster with 80 μg of antigen, dissolved in 200 μl of an emulsion consisting of PBS and incomplete Freund's adjuvant, was given intraperitoneally. A final booster with 80 μg of antigen dissolved in PBS was given another two weeks later. Three days later, the spleen was removed and the lymphocytes were used for fusion.

For cell fusion the mouse myeloma cell line NS-1 was used. These myeloma cells were grown as a batch culture in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), kanamycin (50 μg ml^{-1}), penicillin (10 U ml^{-1}), streptomycin (10 μg ml^{-1}) and 10% (v/v) of heat-inactivated bovine calf serum. PEG-induced cell fusion was performed as described by van Duijn *et al.* (1989). About 10 d after the fusion, the antibody production of the hybridomas was tested using an ELISA system. The (anti-catalase)-antibody-producing cells were harvested and diluted in the selective medium and seeded over another 96-well tissue culture plate so that each well contained one cell. Before the cell-lines were considered to be monoclonal, this so-called cloning procedure was repeated four times with intervals of approximately two weeks.

To obtain sufficient amounts of monoclonal antibodies, the cloned cell-lines were transferred to 50 ml cell culture flasks and cultured in standard medium (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) bovine calf serum) for 2-5 d. Subsequently, these cells were cultured in 260 ml flasks for 4-10 d. The monoclonal antibodies secreted into the medium by the hybridomas were purified using protein G affinity column chromatography.

The production of antibodies of the IgG isotype was tested by using an ELISA system as described by de Boer *et al.* (1988), with the modification that goat anti-(mouse IgG/Fc) immunoglobulins conjugated to alkaline phosphatase were used. Bound alkaline phosphatase activity was revealed by the addition of 100 μl of a substrate solution (1 mg p-nitrophenyl phosphate per ml and 2 mM $MgCl_2$ in 0.1 M diethanolamine (pH 9.8)). After 1 h at 37°C colour development was stopped by the addition of 100 μl of 0.3 M glycine/NaOH (pH 10.6) per well. Extinction was

determined spectrophotometrically at 405 nm with a Titertek Multiscan MC.

Immunocytochemistry. For immunocytochemical localization of catalase, tissue slices smaller than 1 mm^2 from barley leaves or aleurone layers, were fixed for 2 h in 0.1 M sodium cacodylate buffer (pH 7.2), containing 2% glutaraldehyde, washed, dehydrated and embedded in LR White Resin (Craig and Miller, 1984). Ultrathin sections were cut with a diamond knife using a Reichert/Jung Ultra-cut, mounted on uncoated nickel grids, washed with distilled water and PBST and then floated on drops of PBST containing 1% BSA for 10 min.

After washing with PBST, sections were incubated for 2 h with catalase-specific monoclonal antibodies, diluted 1:5,000-1:10,000 in PBST containing 0.1% BSA. Subsequently, the sections were incubated for 90 min with protein A gold particles, diluted 30 times in the same buffer. After several rinses with PBST and distilled water, sections were stained with 1% aqueous uranyl acetate for 10 min and examined with a Philips 300 electron microscope at 60 kV. Control experiments were performed by omitting the antibody incubation step, or by replacing the catalase-specific antibodies by antibodies directed against barley α-amylase-2.

Cytochemistry. Catalase activity was localized by a modification of the method of Novikoff and Goldfischer (1969). Tissue slices, as described above, were fixed in 0.1 M sodium cacodylate buffer (pH 7.2), containing 2% para-formaldehyde and 2.5% glutaraldehyde for 2 h at 0°C. Subsequently, the slices were preincubated in 0.1 M Tris-HCl buffer (pH 9.0) containing 1 mg ml^{-1} DAB for 1 h at 25°C in the dark.

Incubation was performed in the same medium supplemented with 0.1% H_2O_2 for 3 h at 25°C in the dark. The incubation medium was refreshed every hour. Control experiments were performed by adding 50 mM AT, 10 mM KCN or 1 mM NaN_3 to preincubation and incubation medium. In other controls DAB or H_2O_2 was omitted. The tissue slices were post-fixed overnight in 0.1 M sodium cacodylate buffer (pH 7.2), containing 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Epon 21045. Ultrathin sections, cut as described above, were examined with a Philips 300 electron microscope.

RESULTS

Enzyme purification and characterization

Leaves from 6 day old barley seedlings contained significant levels of catalase activity and were used as start-up material for purification, as detailed in table 1.

Table 1. Summary of the purification of barley leaf catalase

Purification step	Enzyme activity (*μkat*)	Enzyme recovery (%)	Protein content (*mg*)	Specific activity (*μkat mg^{-1}*)	Purification (*-fold*)
Homogenate	4967	100.0	24902	0.2	1
PBE 94	2685	54.1	507	5.3	26
Sephacryl S-300	2372	47.8	31	76.5	382
Mono P	840	16.9	1.74	482.8	2414
Superose 12	163	3.3	0.43	379.1	1895

The presence of catalase in the purified fraction was confirmed by the following observations. First, the ratio of absorption at 280 and 405 nm of the purified preparation was 2.38, indicating the presence of a heme-containing protein. Second, after SDS-PAGE and Western blotting of the obtained preparation, followed by incubation with an antiserum against maize catalase-1, a 55 kDa band reacting with this antiserum was observed (Fig. 1).
Finally, after native PAGE, followed by staining for catalase activity with DAB, a clear activity band was observed (Fig. 2), which was absent in controls with AT.

The obtained preparation is not entirely pure. The ratio of absorption at 280 and 405 nm is higher than the values of 1.5 and 1.0 observed for other plant catalases (summarized by Yamaguchi and Nishimura, 1984), indicating some contamination with other proteins. Furthermore, after native PAGE followed by activity staining with DAB, only one of the three silver-stained bands showed catalase activity (Fig. 2).

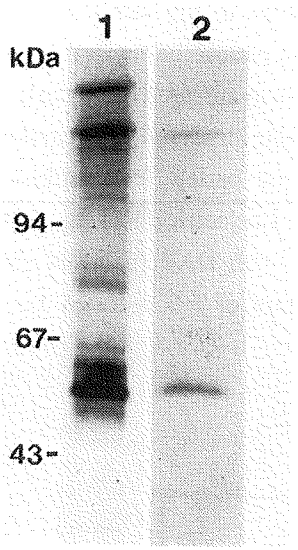

Figure 1. Protein composition and cross-reaction with maize catalase antibodies of the purified barley leaf catalase preparation. SDS-PAGE performed on a homogeneous 12.5% PhastGel was followed by either silver staining (lane 1, 80 ng of protein) or Western blot analysis, using polyclonal antibodies against maize catalase-1 (lane 2, 800 ng of protein). Molecular mass markers are indicated on the left.

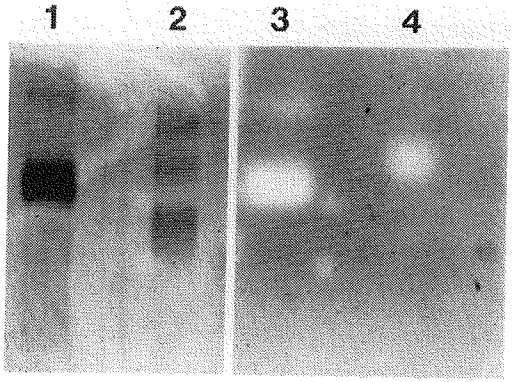

Figure 2. Protein composition and activity staining of the purified barley leaf catalase preparation. Native PAGE of purified leaf catalase (lanes 2 and 4; 100 ng of protein) and of bovine liver catalase as a control (lanes 1 and 3; 100 and 50 ng of protein respectively) was performed on a homogeneous 12.5% PhastGel. The gel was either silver-stained (lanes 1 and 2) or stained for catalase activity with DAB (lanes 3 and 4).

By sedimentation velocity centrifugation the native molecular mass of catalase was estimated to be 245 kDa (Fig. 3), which is close to the value of 230 kDa determined by Kendall *et al.* (1983) for catalase from mature barley leaves by gel-filtration chromatography. Since SDS-PAGE gave a subunit molecular mass of 55 kDa, it is likely that barley leaf catalase has a tetrameric structural organization. By means of chromatofocusing the isoelectric point of barley leaf catalase was determined to be 6.0. This result was confirmed by isoelectric focusing, followed by Western blotting or staining for catalase activity with DAB (data not shown). This isoelectric point is slightly higher than the value of 5.5 reported for lentil catalase (Schiefer *et al.*, 1976).

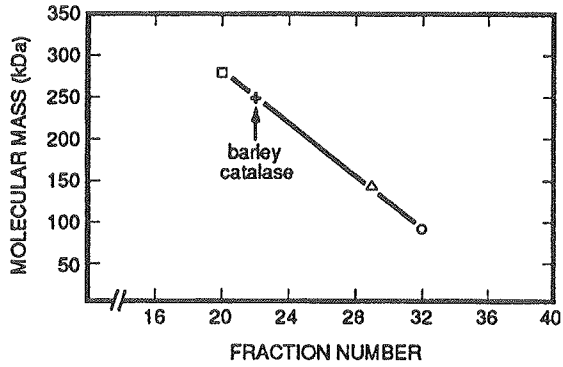

Figure 3. Determination of the native molecular mass of barley leaf catalase by sedimentation velocity centrifugation. (□), xanthine oxidase (275 kDa), (△), lactate dehydrogenase (140 kDa), and (○), citrate synthase (87 kDa) were used as internal markers.

The pH optimum of barley catalase was dependent on the buffer system used. In potassium phosphate buffer catalase showed a pH optimum over a range of 6.0-8.0, whereas in Tris-HCl buffer optimal activities were measured at pH 8.0-9.5 (Fig. 4). The maximum activity measured in Tris-HCl buffer was only 66% of that in potassium phosphate buffer.

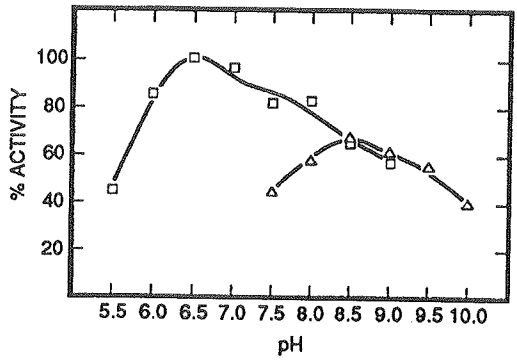

Figure 4. Dependence of the pH optimum of barley catalase on the buffer system used. (□), 0.1 M potassium phosphate buffer; (△), 0.1 M Tris-HCl buffer. The ordinate shows the activity relative to that in potassium phosphate buffer at pH 6.5.

Monoclonal antibodies

Following immunization with the partly purified catalase from young barley leaves, 150 x 10^6 murine spleen lymphocytes were used for fusion with the NS-1 myeloma cells. After polyethyleneglycol-induced hybridization, the lymphocytes were seeded over 1440 wells of 15 microtitre plates. After repeated screening (ELISA) and cloning, seven monoclonal (anti-catalase) antibody-producing hybridomas were obtained. However, three of these seven catalase-positive hybridomas produced antibodies which were also cross-reactive in an ELISA assay with an unrelated protein like (1-3)(1-4)-ß-glucanase-2 (data not shown). As a result, four stable cell-lines (39.3, 39.5, 39.10 and 39.14) were obtained which produced monoclonal antibodies which specifically recognized the purified catalase preparation.

After purification the monoclonal antibodies were further characterized with respect to their antigen-specificity using Western blotting experiments. This is

particularly relevant since the purified catalase preparation showed three major bands on SDS-polyacrylamide gels (Fig. 1). After SDS-PAGE of crude leaf extracts, followed by Western blotting, all four monoclonal antibodies showed a pronounced reactivity with a 55 kDa protein, most likely representing a subunit of barley leaf catalase (illustrated for cell lines 39.3 and 39.14 in Fig. 5).

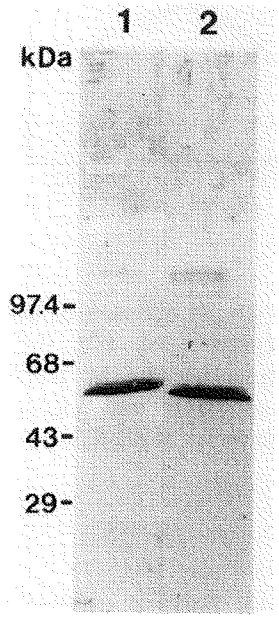

Figure 5. Recognition of catalase subunits from crude leaf extracts by monoclonal antibodies. A crude extract of barley leaves (30 μg of protein) was separated on a 10% SDS-polyacrylamide gel using the Midget system, followed by Western blotting and incubating with monoclonal antibodies from cell lines 39.3 (lane 1) and 39.14 (lane 2). Molecular mass markers are indicated on the left.

Often in these Western blots also two weakly stained high-molecular-mass bands (> 97.4 kDa) were observed. These two bands were also detected in Western blots of the purified preparation (Fig. 6) and are probably related to multimeric or aggregated forms of catalase subunits. The specificity of the monoclonal antibodies was substantiated after subjecting purified leaf catalase to native PAGE. One part of the gel was stained for catalase activity with DAB, whereas the other part was blotted onto nitrocellulose and incubated with monoclonal antibodies from each of the four cell lines. DAB-staining revealed the presence of a single activity band, at a position comparable to that of an immunoreactive protein band on the corresponding Western blot (illustrated for cell-line 39.3 in Fig. 7, lanes 1 and 5). These results indicate that the four monoclonal antibodies are catalase-specific.

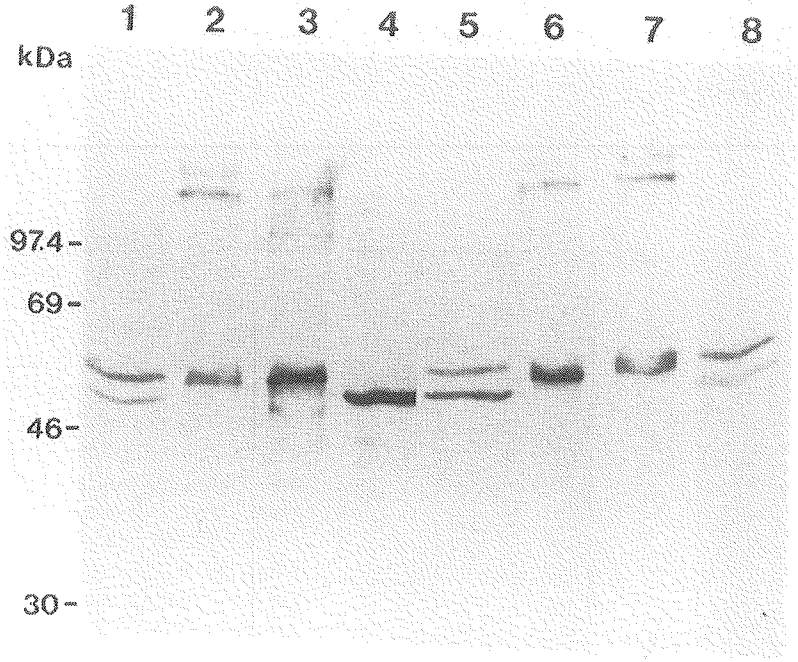

Figure 6. Recognition of purified leaf catalase subunits and catalase subunits from crude aleurone extracts by monoclonal antibodies. Purified leaf catalase (lanes 2, 3, 6 and 7; 0.8 μg of protein) and a crude extract of aleurone layers (lanes 1, 4, 5 and 8; 52 μg of protein) were electrophoresed on a 10% SDS-polyacrylamide gel using the Midget system, followed by Western blotting and incubating with monoclonal antibodies from cell lines 39.3 (lanes 1 and 2), 39.5 (lanes 3 and 4), 39.10 (lanes 5 and 6) and 39.14 (lanes 7 and 8). Molecular mass markers are indicated on the left.

The four monoclonal antibodies have been tested for their possible cross-reactivity with catalase present in aleurone cells. For this purpose proteins extracted from barley aleurone layers were separated by SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. As can be seen in figure 6 (lanes 1, 5 and 8), three monoclonal antibodies (39.3, 39.10 and 39.14) show cross-reactivity with two protein bands on these Western blots. The upper band represents a protein with a molecular mass of approximately 57 kDa, which is

slightly higher than that of the barley leaf catalase subunit. Furthermore, an immuno-reactive protein with a molecular mass of 51 kDa, which is less than that of leaf catalase, can be observed on these Western blots. Interestingly, monoclonal antibody 39.5 recognizes exclusively this 51 kDa protein band on Western blots (Fig. 6, lane 4)

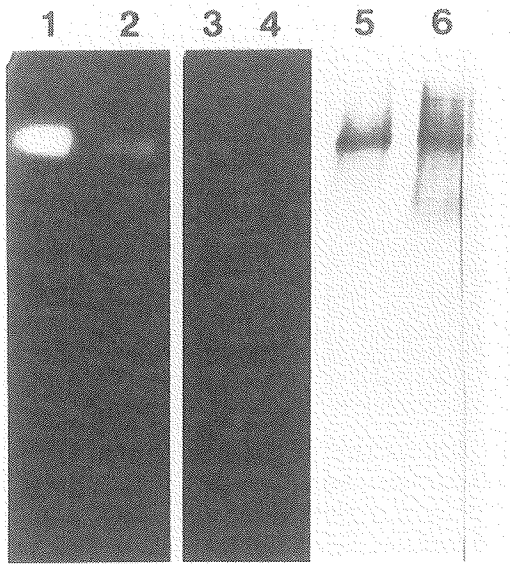

Figure 7. Recognition of native catalase from the purified leaf preparation and crude aleurone extracts by monoclonal antibodies. Purified leaf catalase (lanes 1, 3 and 5; 3.3 μg, 3.3 μg and 1.6 μg of protein respectively) and a crude extract of aleurone layers (lanes 2, 4 and 6; 52 μg of protein in each lane) were electrophoresed on a 7.5% native polyacrylamide gel using the Midget system. Part of the gel was directly stained for catalase activity with DAB (lanes 1-4), either in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of AT. The other part of the gel was blotted onto a nitrocellulose membrane and incubated with monoclonal antibodies from cell line 39.3 (lanes 5 and 6).

When extracts of barley aleurone cells were subjected to native PAGE, followed by DAB-staining for catalase activity or Western blotting two immuno-reactive proteins could be detected with each of the four monoclonal antibodies (illustrated for cell line 39.3 in Fig. 7). Activity bands at similar positions on the corresponding DAB-stained part of the gel confirmed that the immuno-reactive products represent catalase. These results show that the four monoclonal antibodies raised against barley leaf catalase are cross-reactive with catalase subunits present in aleurone cells.

Electron microscopy

Next we investigated whether the antibodies generated against catalase could be applied to immunocytochemical visualization of microbodies. After immunogold labelling carried out on ultrathin sections of young leaves and aleurone layers, using a mixture of monoclonal antibodies 39.3 and 39.14, specific labelling was exclusively found in the microbody matrix (Fig. 8 A and B).

Labelling of microbodies in the aleurone layer was concentrated in the electron dense core of these organelles. In the leaf microbodies, which lacked such a dense core, labelling was present throughout the matrix. The density of gold labelling in the leaf microbodies was 57 gold particles per μm^2, compared to 0.5 for other cell compartments. For the aleurone these figures were 99 and 1.2 respectively. Similar results were obtained when antibodies from cell lines 39.3, 39.10 and 39.14 were used separately for immunolabelling (data not shown). No labelling was observed with antibodies from cell line 39.5. The cause for the aberrant behaviour of these antibodies in immunocytochemistry is unknown. In control experiments in which the catalase-specific antibodies were omitted or replaced with antibodies directed against barley α-amylase-2, no labelling of microbodies was observed. Further immunocytochemical experiments revealed that also in the scutellum labelling of catalase is restricted to microbodies (data not shown). In Fig. 8 the membrane surrounding microbodies is hardly visible. This relatively poor preservation of the ultrastructure is due to the use of low fixative concentrations required for immunocytochemistry.

The location of catalase in microbodies was confirmed by cytochemical staining of catalase activity with DAB. Examination of sections of young leaves and aleurone layers revealed a clear staining of the microbody matrix, the cristae of the mitochondria and, in the case of leaves, of the chloroplast thylakoids (data not shown). In the presence of 50 mM AT, a concentration that inhibits all catalase activity in extracts of leaves and aleurone, no DAB-staining was observed in microbodies. However, in mitochondria and chloroplasts, the DAB oxidation product was still apparent. These results demonstrate that staining of the microbodies is a result of catalase activity. Staining of the mitochondria and the chloroplasts may be due to cytochrome oxidase and peroxidase activity respectively (Frederick, 1987). In all other controls, i.e. in the presence of KCN or NaN_3, or in the absence of DAB or H_2O_2, no reaction product could be detected.

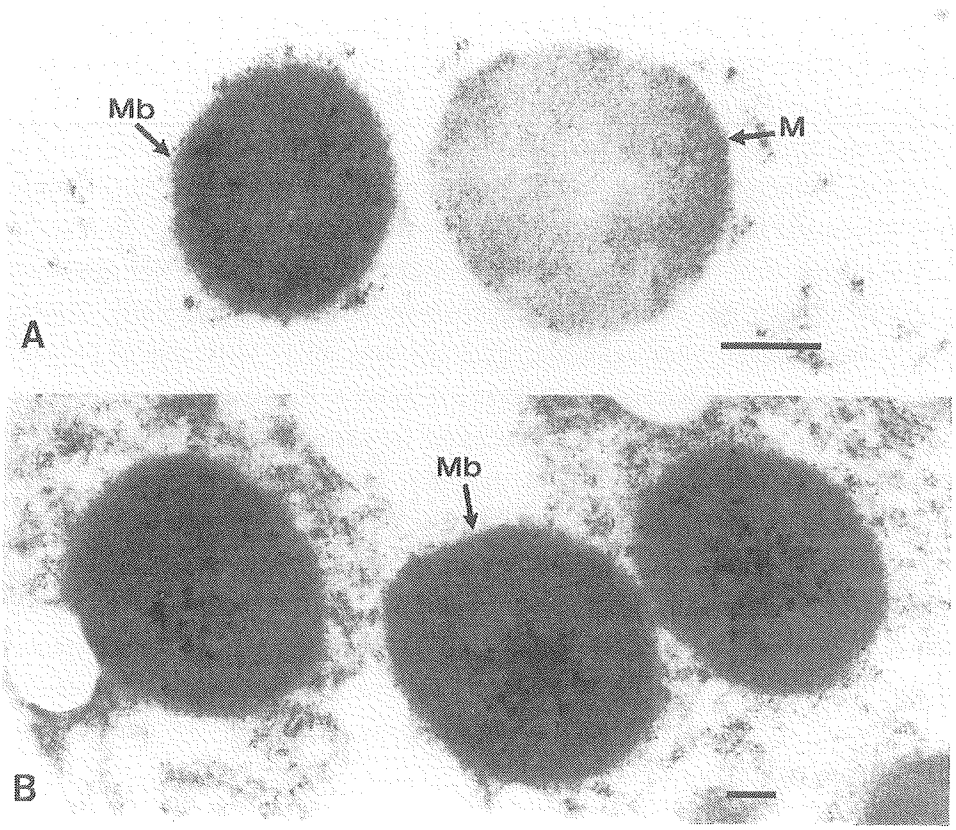

Figure 8. Immunogold labelling of catalase in young leaves (A) and aleurone (B). Immunogold labelling was performed using a mixture of monoclonal antibodies from cell-lines 39.3 and 39.14. Abbreviations: Mb, microbody; M, mitochondrion. The bar represents 0.2 μm.

DISCUSSION

So far one structural gene for catalase, *Cat1* on barley chromosome 4 has been identified by analyzing the electrophoretic patterns of catalase isoenzymes in 7 day old seedling leaves of wheat barley chromosome addition lines (Benito *et al.*,

1985). It is not known whether the catalase deficient barley mutant RPr 79/4 (Kendall *et al.*, 1983) is due to a mutation in this gene, but it may well turn out that the barley mutation has occurred in the *Cat1* gene on chromosome 4, since the electrophoretic zymogram of the wheat barley chromosome 4 addition line seedlings revealed a single sharp barley catalase band in addition to the multiple bands encoded by the wheat genomes.

Kendall *et al.* (1983) noticed that leaves in the catalase deficient barley mutant still contained 10% of the catalase activity found in the wild type. A phenocopy of the mutant with a reduction of catalase activity by almost 70% is obtained by growing wild type seedlings in the dark (Lea *et al.*, 1992). This is taken as evidence that barley as other plants produces at least two isoenzymes of catalase. In this paper the occurrence of three catalase subunits in germinating barley is described. Differences are observed in the electrophoretic mobility of the three subunits, the young leaf subunit moving with a molecular mass of 55 kDa, those of the aleurone corresponding to 57 and 51 kDa respectively. Monoclonal antibody 39.5 recognizes an epitope which is present in the low molecular mass ($\approx$ 51 kDa) catalase subunit of aleurone cells (Fig. 6, lane 4), but apparently is absent from the 57 kDa subunit of these cells. The epitope is also present in the single subunit ($\approx$ 55 kDa) recognizable in leaf cells.

Monoclonal antibodies 39.3, 39.10 and 39.14 recognize an epitope which is shared by the subunit of leaf cells and both subunits present in aleurone cells. It will be of interest to analyze to what extent the leaf and two aleurone subunits are homologous to the polypeptides identified in maize and pumpkin. This requires cDNA cloning of the barley and pumpkin structural genes for catalase. The monoclonal antibodies characterized in the present study will be helpful in assigning the barley subunits revealed by electrophoresis to the *cat1* gene and putative additional genes.

The relation between the two catalase subunits in aleurone is unknown. We investigated if the 51 kDa form could be a proteolytic modification (Kindl, 1982) of the 57 kDa polypeptide by testing, if isolation of the catalase subunits from aleurone layers in the presence of protease inhibitors such as bacitracin, EDTA-Na_2, leupeptin, pepstatin and PMSF could prevent the occurrence of the 51 kDa band. This was not the case. Processing of catalase precursors inside microbodies has been suggested by Yamaguchi *et al.* (1984) for pumpkin and by Eising *et al.*

(1990) in sunflower, but it is unknown whether this could be the case for barley aleurone too. However, the observation that antibodies from cell line 39.5 only recognize the smaller of the two catalase subunits may suggest that there is no precursor-product relationship between the two polypeptides.

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Chapter 4

The ratio of peroxidative to catalatic activity of catalase in leaves of barley seedlings is linked to the expression levels of two catalase subunits

Submitted

The ratio of peroxidative to catalatic activity of catalase in leaves of barley seedlings is linked to the expression levels of two catalase subunits

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Peter J. Lea and Jan W. Kijne

ABSTRACT

Function and regulation of expression of an enhanced (EP-CAT) and normal (T-CAT) peroxidative form of catalase were studied in leaves of young barley (*Hordeum vulgare* L.) seedlings. A catalase-deficient mutant and the wild type were grown either in the dark, shade or light and assayed for peroxidative and catalatic activities of catalase. Additionally, the subunit pattern of catalase was studied, using catalase-specific monoclonal antibodies. A high ratio of peroxidative to catalatic activity of catalase was found in the dark, which was linked to induction of a 57 kD catalase subunit. Increased levels of a 53 kD subunit were detected after growth in the light, which was linked to normal levels of peroxidative to catalatic activity. We show that the EP-CAT and the T-CAT forms of catalase are regulated by expression of the 57 and 53 kD subunits respectively. The catalase-deficient mutant synthesizes the 53 kD subunit in limited amounts, but fails to upregulate its expression upon transfer of the seedlings to high light intensity. Interestingly, grains of the catalase-deficient mutant showed a higher germination index than those of the wild type. Addition of hydrogen peroxide to wild type grains resulted in a germination index which was close to that of the mutant, suggesting that catalase activity levels at the onset of germination are related to the rate of germination.

Keywords: *Hordeum vulgare*, catalase subunits, peroxidative- and catalatic activity, regulation

Abbreviations: CAT, catalase protein or enzyme; *Cat*, catalase gene or transcript; EP-CAT, enhanced peroxidative activity catalase; Mab, monoclonal antibody(ies); T-CAT, normal peroxidative activity catalase

INTRODUCTION

The enzyme catalase (EC 1.11.1.6) is a tetrameric, heme-containing enzyme which is present in all aerobic organisms. It plays an important role in the decomposition of hydrogen peroxide, a toxic intermediate of oxygen metabolism.

In plants, multiple forms of catalase have been found both in dicots, such as cotton, tobacco, soybean, castor bean, mung bean, cotton, pea, tomato, sunflower, sweet potato and *Arabidopsis*, and in monocots, such as maize, rice and barley (summarized by Scandalios, 1994; Willekens *et al.*,1995; Skadsen *et al.*, 1995).

Based upon expression properties, plant catalases have been classified into three distinct classes, each exhibiting a specific cellular function (Willekens *et al.*, 1995). The first class is abundantly expressed in the peroxisomes of photosynthetic tissues. In these tissues, catalase neutralizes H_2O_2 which is generated during the conversion of glycolate, produced in the chloroplasts during photorespiration, into glyoxylate (Zelitch and Ochoa, 1953). The second class of catalases is found in glyoxysomes of lipid storing tissues, where it decomposes H_2O_2, which is formed during acyl-CoA oxidation of fatty acids (Yamaguchi *et al.*, 1984; Eising and Gerhardt, 1987). The third class of catalases, present in vascular tissues, has been suggested to play a role in the protection against environmental stress (Willekens *et al.*, 1994). Catalase is possibly involved in the signaling pathway of H_2O_2 (Chen *et al.*, 1993), which in turn was suggested to play a central role in hypersensitive response in disease resistance (Tenhaken *et al.*, 1995). Although this classification was primarily described for dicot catalases, it may, because of functional similarities between dicot and monocot catalases, hold for monocot catalases as well.

The catalases of maize, barley and rice have been classified by homology between amino acid sequences into two distinct groups (Skadsen *et al.*, 1995). The first group of monocot catalases shows a high homology with the mitochondria-associated CAT-3 of maize, which is preferentially expressed in the leaves of dark-

grown maize (Redinbaugh *et al.*, 1990), while the second group is homologous to the peroxisomal/glyoxysomal maize CAT-1.

Catalase forms with different catalytic properties have been described for barley, maize and tobacco, one catalase form exhibiting enhanced peroxidative activity (EP-CAT), the other showing "normal" peroxidative activity (T-CAT). The activity of T-CAT is light dependent whereas that of EP-CAT is not (Havir and McHale, 1989b). In barley and tobacco, the EP-CAT form has 30-fold higher peroxidative activity than the T-CAT form, while in maize the EP-CAT form exhibits a 70-fold higher peroxidative activity than the T-CAT form.

EP-CAT and T-CAT forms have been shown to be under separate genetic control. That is, in maize the EP-CAT form is the product of the *Cat3* gene, which is expressed only in mesophyll cells of dark grown maize (Tsaftaris *et al.*, 1983), whereas *Cat2* encodes the T-CAT form (Havir and McHale, 1989b). Also in tobacco and barley, T-CAT and EP-CAT are encoded by different genes. Thus, *Cat1* and *Cat3* code for respectively T-CAT and EP-CAT in tobacco (Havir and McHale, 1989b), while in barley EP-CAT and T-CAT are encoded by *Cat1* and *Cat2* respectively (Acevedo *et al.*, 1996).

Although regulation of expression of EP-CAT and T-CAT forms of catalase has been elucidated at the levels of activity and mRNA, it remained to be studied how both catalytic forms are regulated at the protein level. Also the exact roles of the EP-CAT and the T-CAT forms in plant metabolism are unknown. In maize, the null-lines for both the CAT-2 and CAT-3 form, still grow normally in air and are fully fertile, which indicates the compensating activity of the CAT-1 form. However, a barley mutant in which catalase activity in leaves of mature plants was found to be less than 10 % of that of the wild-type, grows poorly in normal air and can only survive under conditions suppressing photorespiration, i.e. low light or high levels of CO_2 (Kendall *et al.*, 1983; Parker and Lea, 1983). This reduction in catalase activity, which is caused by the lack of expression of the T-CAT form (Havir and McHale, 1989b; Acevedo *et al.*, 1996), underlines the importance of the T-CAT form for normal plant growth. For tobacco, different forms of catalase were separated after chromatofocusing. A form with low peroxidative activity has been suggested to be involved in photorespiration, while a form with enhanced peroxidative activity which was induced under CO_2-enrichment, was suggested to be unrelated to photorespiration (Havir and McHale, 1987)

The availability of catalase-specific monoclonal antibodies, enabled us to study the function as well as the regulation of expression at the protein level of the enhanced peroxidative (EP-CAT) and the "normal" (T-CAT) forms of catalase in leaves of germinating barley. Seedlings of the above-mentioned barley mutant, which was suggested to lack expression of the T-CAT form, and the parental variety were grown under different light conditions and were subsequently assayed for catalatic and peroxidative activities. The monoclonal antibodies which were used to follow protein levels of catalase, have been shown to recognize differentially catalase subunits in barley aleurone cells and leaves (Holtman *et al.*, 1993)

Our results show that the expression levels of the two catalase subunits in barley leaves are determined by the light conditions and that these levels are correlated with the ratio of peroxidative to catalatic activity of catalase. The possible functions of T-CAT and EP-CAT in germinating barley are discussed. Additionally, differences between grains of the catalase-deficient mutant and wild type grains in the rate of germination will be described.

MATERIALS AND METHODS

Plant materials. The catalase-deficient mutant of barley LaPr 86/85 was isolated from an azide mutated population of barley *Hordeum vulgare* L. cv Maris Mink by R.D. Blackwell, A.J. Murray and P.J. Lea at Lancaster University. The properties of the mutant line were shown to be very similar to the original catalase deficient mutant line RPr 79/4 isolated at Rothamsted (Kendall *et al.*, 1983; Parker and Lea, 1983).

Grains from the catalase deficient mutant and its parental line Maris Mink were propagated at 15 °C. The mutant was germinated and grown under low light conditions, i.e. a photoperiodic cycle of 8 h dark and 16 h at approximately 80 μmol m^{-2} s^{-1}, while the wild-type grains were germinated and grown with the same photoperiod at 320 μmol m^{-2} s^{-1}. Grains from selfed grains were used for the experiments.

In all experiments described, germination of the grains was performed at 15 °C in a plastic plant propagator between two times three layers of moistened filter

paper. After 2 days, filter paper on top of the seedlings was removed. Three different light regimes were used: A: continuous darkness, B: alternately 8 h dark and 16 h 80 μmol m^{-2} s^{-1}, C: alternately 8 h dark and 16 h 320 μmol m^{-2} s^{-1}. Henceforth these conditions will be called "dark", "shade" and "light", respectively.

When plants older than 11 days were used, seedlings were transferred into pots after 6 days. Leaves (coleoptile included) were harvested from plants at the days indicated.

Enzyme assays and protein determination. The catalatic activity of catalase was assayed according to the spectrophotometric method of Lück (1963). One Unit is defined as the amount of enzyme decomposing one μmol H_2O_2 per minute.

The peroxidative activity of catalase was determined spectrophotometrically, by measuring the formation of acetaldehyde with ethanol as a substrate, by a modification of the method of Havir and McHale (1989a). Briefly, D-glucose (16 mg ml^{-1}) was converted into gluconate and hydrogen peroxide by glucose oxidase (3.18 Units ml^{-1}; Boehringer, Mannheim, Germany). Hydrogen peroxide was subsequently reduced by catalase with simultaneous oxidation of ethanol. The amount of acetaldehyde produced, was followed by the formation of NADH from NAD (1.0 mg ml^{-1}) with aldehyde dehydrogenase (0.6 Units ml^{-1}; Boehringer, Mannheim, Germany) at 340 nm. The reaction was carried out in a 1 ml cuvette at pH 7.0 in a 50 mM potassium phosphate buffer; final concentration of components in the reaction mixture are indicated between brackets. The reaction was started with the addition of ethanol (55 μl, 96 %). One unit is defined as the amount of enzyme catalyzing the formation of 1 μmol acetaldehyde per minute. The ratio of peroxidative to catalatic activity of catalase is defined as mU peroxidative: U catalatic.

Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay, using BSA as a standard (Smith *et al.*, 1985).

Preparation of crude extracts. All steps were performed at 0 to 4 °C. Crude extracts were prepared by homogenizing 10 leaves in 20 mM potassium phosphate

buffer (pH 7.0), containing 1 mM $MgCl_2$ and 0.5 mM PMSF. For extraction the ratio of buffer (ml) to fresh weight (g) was 10:1, with a minimum of 2 ml. After homogenization using an Ultra Turrax (Janke & Kunkel, Staufen, Germany)(five pulses at 24,000 rpm of 30 s each, with 30 s intervals) the resulting suspension was centrifuged for 5 min at 3,400 g. Subsequently, the supernatant was centrifuged at 15,800 g for 20 min. The supernatant was assayed for catalase activity and stored for further use at -20 °C.

Gel electrophoresis and Western blotting. Generally, SDS-PAGE was performed on 12.5 % homogeneous gels using the Phast System (Pharmacia Biotech, Uppsala, Sweden). For determination of the molecular mass of catalase subunits, extracts were electrophoresed using the excel gel system, from the same manufacturer.

After electrophoresis, proteins were transferred onto nitrocellulose membranes by semidry blotting essentially as described by Towbin *et al.* (1979) and Kyhse-Andersen (1984). After blotting, the membranes were soaked in phosphate buffered saline containing 0.05 % Tween 20 (PBST) plus 1 % BSA for 30 min, followed by incubation overnight with catalase specific Mab (cell-lines 39.14 and 39.5), diluted in the same buffer. These Mab (kindly provided by Dr. G. van Duijn, Netherlands Organization for Applied Scientific Research, Nutrition and Food Research Institute, Division of Biochemistry and Gene Technology, Zeist, The Netherlands) showed differential recognition of catalase subunits in barley aleurone cells and leaves (Holtman *et al.*, 1993). That is, Mab from cell-line 39.14 recognized a 55 kD subunit in barley leaves as well as 51 and 57 kD subunits in aleurone cells. Mab from cell-line 39.5 recognized the leaf 55 kD catalase subunit, and the 51 kD aleurone subunit, but not the 57 kD subunit. Subsequently, the membranes were washed with PBST and incubated for 1 h with goat anti-mouse IgG antibodies conjugated to alkaline phosphatase. Finally, the bound phosphatase was visualized by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as the substrate. Colour development was stopped by washing the membranes with distilled water.

Germination tests. Hundred grains from the catalase deficient mutant LaPr 86/85 or its parental line Maris Mink were germinated in a 9 cm Petri-dish with 2 layers of filter paper (Schleicher & Schuell) and 4 ml of distilled water at 20°C in the dark.

The germination energy (GE) is expressed as the percentage of grains germinated after 72 h. The germination index (GI) was calculated according to a relative scale, where more value was given to fast geminating grains by a modification of the method of Walker-Simmons (1987). GI= 10 x total amount of grains germinated after 3 days divided by the sum of those germinated after 24 h, 48 h and 72 h (those germinated after 24 h times one, those germinated after 48 h times two and those germinated after 72 h times three). In certain germination tests, grains were germinated for 24 h on filter paper moistened with 4 ml distilled water containing 2.10^{-3} M hydrogen peroxide, and then transferred to filter paper moistened with distilled water. As a control in these experiments, germination was performed on filter paper containing distilled water only.

RESULTS

Catalase activity in leaves of germinating barley

In order to follow the expression of catalase during germination of barley grains, we assayed catalatic catalase activity in wild type seedlings as well in catalase-deficient mutant seedlings. Because the mutant could only survive under conditions suppressing photorespiration, these seedlings were grown in the shade. Wild-type seedlings were grown both in the shade and in the light.

In leaves of wild-type seedlings, grown in the light, catalase activity strongly increased after 5 days of germination, probably reflecting the need to degrade hydrogen peroxide as a result of photorespiration (Fig. 1). The temporal decrease in activity found after day 7 may reflect the reduction of catalase-specific activities which occurs during the transition of microbodies (from glyoxysomes to leaf peroxisomes) during the greening process, as reported for fat storing dicotyledonous seedlings (Trelease *et al.*, 1971; Kagawa and Beevers, 1975; Yamaguchi *et al.*, 1984). Also in leaves of wild type plants grown in the shade, activity increased

during germination. However, levels were significantly lower than those in leaves grown in the light.

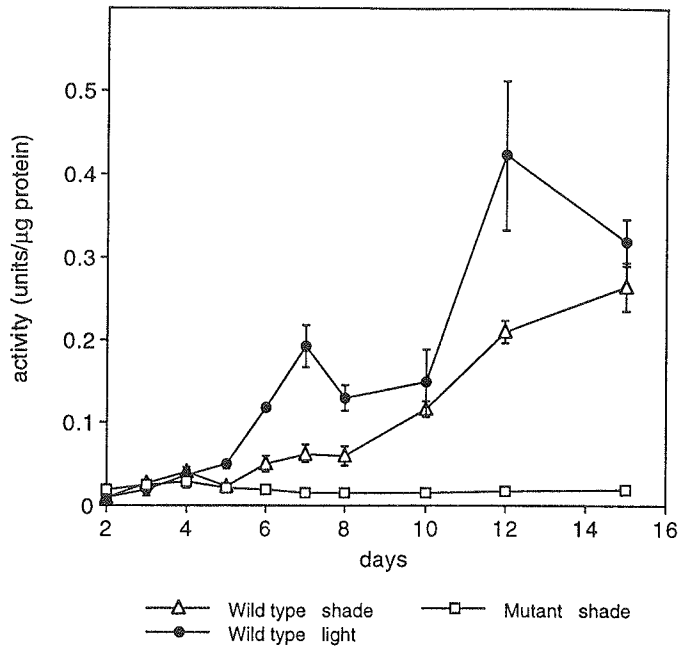

Figure 1. Time course of catalatic catalase activity during germination in leaves of barley seedlings of the wild type, grown in the shade (△) or light (●), and mutant LaPr 86/85 grown in the shade (□). Activity is expressed as units per μg protein. Values are the means ± SE of two independent experiments.

In leaves of the catalase-deficient mutant, low levels of catalase activity were detected, which were constant during the time period. In 15-days-old seedlings, catalase activity was about 10 % of that measured in the leaves of the wild-type which were grown under the same light conditions, i.e. shade. Similar low levels of catalase activity in the mutant line (RPr 79/4) were reported by Kendall *et al.* (1983) in 3 weeks old barley plants, which contained only 10 % of the activity found in the wild type.

Expression of catalase subunits in barley leaves during germination

The expression of catalase was then studied at the protein level. Extracts of leaves from mutant seedlings grown in the shade and wild type seedlings grown in the light, were subjected to SDS-PAGE and Western blotting. Blots were incubated with two different catalase-specific Mab, namely 39.14 and 39.5.

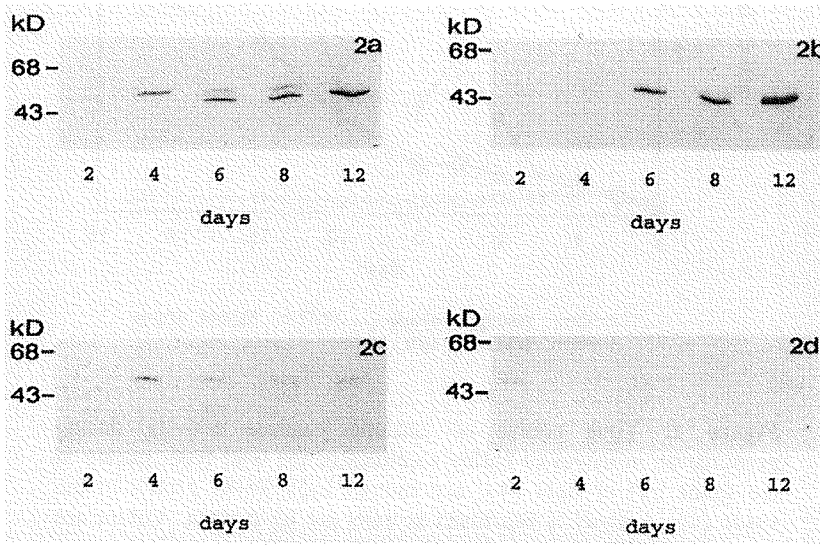

Figure 2. Expression of catalase subunits during germination in leaves of barley seedlings of the wild type (Figs 2a and 2b) and mutant LaPr 86/85 (Figs 2c and 2d). After SDS-PAGE and Western blotting, the blots were incubated with catalase-specific Mab 39.14 (Figs 2a and 2c) or 39.5. (Figs 2b and 2d). Each lane contained 1.9 μg protein. Molecular mass markers are indicated on the left.

As can be seen in Figure 2a, Mab 39.14 recognized in leaf extracts of the wild type two different polypeptides, most probably representing catalase subunits, with estimated molecular masses of 53 kD and 57 kD. On the other hand, Mab from

cell-line 39.5 detected only one polypeptide in these extracts, representing the 53 kD subunit of catalase (Fig. 2b). Large molecular mass bands (» 97.4 kD), which often could be detected on these blots, probably represent multimeric or aggregated forms of catalase subunits (not shown).

Interestingly, on the blot with extracts of the wild type, which were incubated with Mab 39.14 (Fig. 2a), a shift could be observed in the expression pattern of the two protein bands during seedling growth (Fig. 2a). During early germination only the 57 kD band could be detected in these extracts, which gradually decreased over time, whereas from day 6 on the 53 kD band appeared which was the only detectable polypeptide at day 12. When these Western blots were incubated with Mab 39.5, only the 53 kD subunit is detected from day 6 onward (Fig. 2b), which is in agreement with the results using Mab 39.14 in Figure 2a.

Extracts of the mutant were then subjected to SDS-PAGE, followed by Western blotting and incubation with the antibody 39.14. As can be seen in Figure 2c, only the 57 kD protein band could be detected, with, just as in the wild-type, highest expression levels at day 4. With Mab 39.5 only very low levels, if any, of the 53 kD subunit could be detected (Fig. 2d).

These experiments indicate that the induced expression of the 53 kD catalase-subunit in the wild-type from day 6 onward (Figs 2a and 2b), corresponds to the increased activity levels of catalase in the light after day 5 (Fig. 1). This 53 kD subunit may therefore be a product of the *Cat2* gene, which is light-inducible (Acevedo *et al.*, 1996). In the catalase-deficient mutant, which fails to grow normally in the light, this 53 kD catalase subunit is only present at very low levels (Figs 2c and 2d). On the other hand, the 57 kD subunit, which is expressed both in the wild-type and in the mutant during early germination, may represent the *Cat1* gene product. *Cat1* transcript levels were shown to be repressed in the light (Acevedo *et al.*, 1996).

Expression of catalase subunits in relation to peroxidative and catalatic activity

If indeed the 53 kD and 57 kD catalase subunits, as described above, correspond to the *Cat2* and *Cat1* gene products, respectively, their expression levels may be reflected by differences in peroxidative catalase activity. Thus, high levels of the 57 kD catalase subunits may correspond to enhanced peroxidative activity, while

the expression of the 53 kD subunit may be linked to normal peroxidative activity. To test this hypothesis, wild-type and mutant seedlings were grown under three different light conditions, namely dark, shade and light. After 8 days of germination, leaves were harvested and assayed for catalatic and peroxidative activity of catalase. Subsequently, Western blotting experiments were performed to study the expression of catalase subunits under these different light conditions.

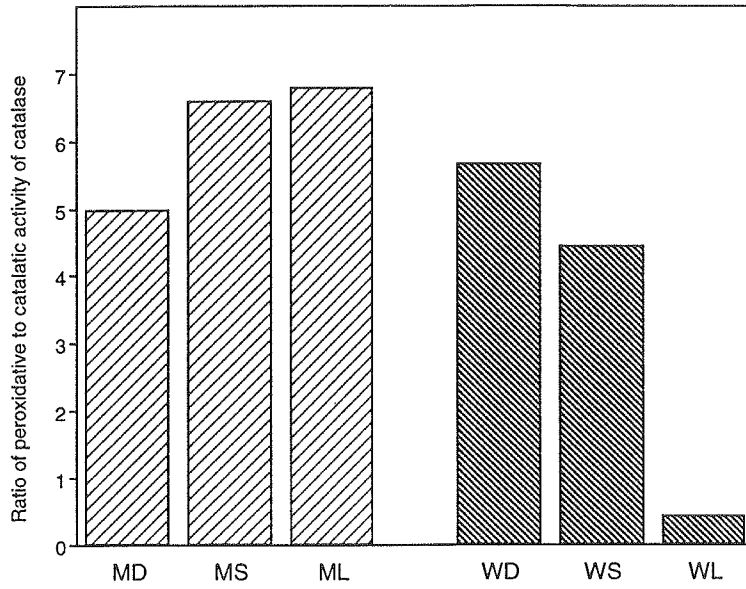

Figure 3. Ratio of peroxidative to catalatic activity of catalase, as defined in Materials and Methods, in leaves of barley seedlings of the mutant LaPr 86/85 (M) and the wild type (W), grown for 8 days in the dark (D), shade (S), or light (L). "MD" means mutant, grown in the dark, and so on.

Figure 3 shows that in leaves of wild-type plants grown in the dark, the ratio of peroxidative to catalatic activity of catalase is significantly higher (by a factor of 14) than in leaves from seedlings which were grown in the light. The higher ratio in leaves from dark-grown seedlings was mainly caused by the peroxidative activity, which was about eight times higher in the dark than in the light. These

differences are reflected by subunit patterns of catalase. As can be seen in Figure 4a, the 57 kD subunit was the predominant subunit in extracts of leaves which were grown in the dark (lane 4), while the 53 kD subunit was the dominant protein band in extracts from leaves which were grown in the light (lane 6). Leaves from seedlings grown in the shade demonstrate an intermediate situation, in which both subunits were present in about equal amounts (lane 5).

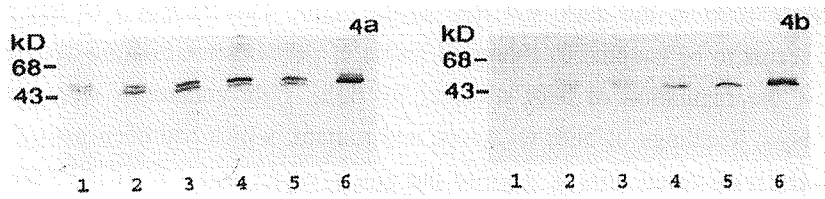

Figure 4. Expression of catalase subunits in leaves of barley seedlings of the mutant LaPr 86/85 and the wild type, grown for 8 days in the dark, shade, or light. After SDS-PAGE and Western blotting, the blots were incubated with catalase-specific Mab 39.14 (Fig. 4a) or 39.5 (Fig. 4b). Lanes 1, 2 and 3: mutant grown in the dark, shade or light, respectively. Lanes 4, 5 and 6: wild type grown in the dark, shade or light respectively. Each lane contained 3.1 μg protein. Molecular mass markers are indicated on the left.

When the same samples were subjected to Western blotting analysis, using Mab 39.5, which can only detect the 53 kD catalase subunit, it can be seen that as the light intensity increased (Fig. 4b, lanes 4 to 6) the intensity of the 53 kD protein band increased as well, emphasizing its light-inducible character.

In the mutant, grown in the dark, shade or light, no significant differences were determined in the ratio of peroxidative to catalatic catalase activity (Fig. 3). In agreement with this, no clear changes were detected in the catalase subunit patterns between the different samples on the corresponding Western blot (Fig. 4a, lanes 1-3). Thus, both subunits were expressed at similar levels under the different growth conditions. With Mab 39.5 only low levels of the 53 kD subunit were detected (Fig. 4b, lane 1-3), indicating again that in the mutant synthesis of this subunit is

hardly induced.

Comparing wild type and mutant, both grown in the light, shows that the significant lower ratio of peroxidative to catalatic activity in the wild type, was mainly caused by a difference in the catalatic activity, which was about 10 times higher in the wild type than in the mutant. This is in nice agreement with data obtained from the germination series (Fig. 1), which showed that catalatic activity at day 15 in the mutant was about 10 % of that in the wild type.

These experiments indicate that the expression of the 53 kD catalase subunit was induced by light and linked to relative low levels of peroxidative catalase activity. On the other hand, the 57 kD catalase subunit showed an opposite regulation, i.e. levels were high in the dark and low in the light. The induction of the 57 kD subunit seems to be coupled to enhanced levels of peroxidative catalase activity.

To test the regulation of both subunits by light, light conditions were changed after 8 days. Seedlings of both wild-type and mutant, which had been grown in the dark, were transferred after 8 days to the shade or the light. Similarly, seedlings first grown for 8 days in the shade were transferred to dark or light, and seedlings which had been grown for 8 days in the light were transferred to dark or shade. In all cases, as a control, seedlings were grown further under the original conditions. After the transfer, the seedlings were grown for another 3 days and leaves from 11 day old seedlings were harvested. Peroxidative and catalatic activities of catalase were measured, while additionally the expression of the different subunits was studied by Western blotting analyses.

Figure 5 shows that when wild-type plants were transferred from the dark or shade into the light, the ratio of peroxidative to catalatic activity of catalase decreased dramatically. The decrease was accompanied by a sharp decrease of expression levels of the 57 kD subunit, while levels of the 53 kD subunit increased under these conditions (Figs 7a and 8a, lanes 1-3). The induction of the 53 kD catalase subunit as a response to light, was also demonstrated by Mab 39.5 (Figs 7b and 8b, lanes 1-3).

When, on the other hand, wild-type seedlings were transferred from the light into the shade or into the dark, the ratio of peroxidative to catalatic activity increased (Fig. 5). On the corresponding Western blot, this increase was accompanied by a slight decrease of the 53 kD subunit to a basic level (Figs 9a and 9b, lanes 1-3). The expression of 57 kD subunit was hardly induced when 8-days-old seedlings

were transferred from the light into the dark (Fig. 9a, lane 1). Apparently, in seedlings which were already grown for 8 days in the light, levels of the 53 and 57 catalase subunits are less inducible, than in seedlings which were grown first in the shade or in the dark.

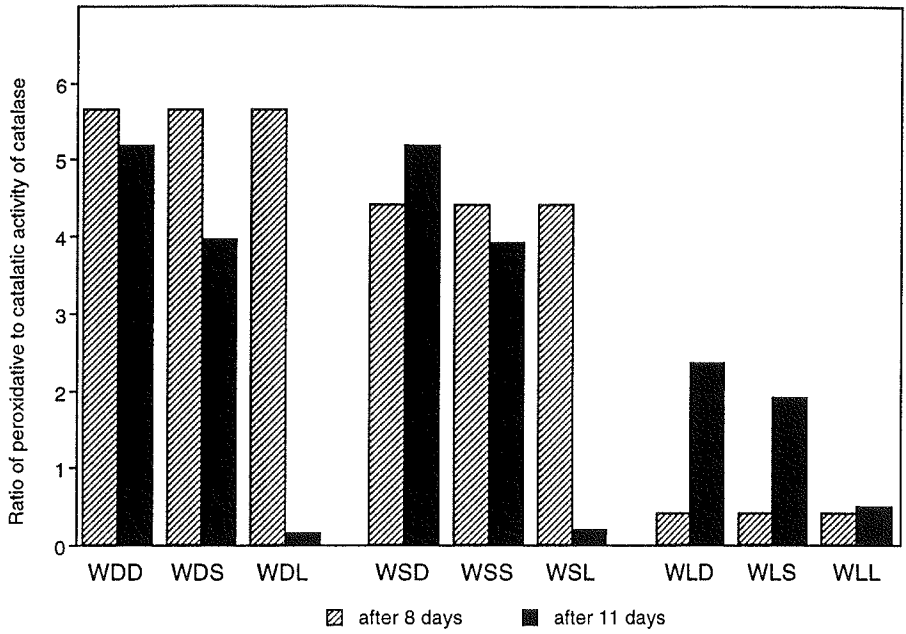

Figure 5. Ratio of peroxidative to catalatic activity of catalase, as defined in Materials and Methods, in leaves of wild type (W) barley seedlings. After a first period of 8 days in the dark (D), shade (S) or light (L), light conditions were changed and the seedlings were grown for another 3 days. For example, "WDL" means wild type, grown first for 8 days in the dark, followed by 3 days in the light.

In the mutant, only minor changes in the ratio of peroxidative to catalatic activity could be detected when the light conditions were changed (Fig. 6). Also, no changes were visualized in the expression of the 53 kD and 57 kD subunits on the corresponding Western blots (Figs 7,8 and 9). Furthermore, in agreement with results obtained with the wild type plants, after 8 days the 57 kD, dark-inducible, catalase subunit could not be induced further.

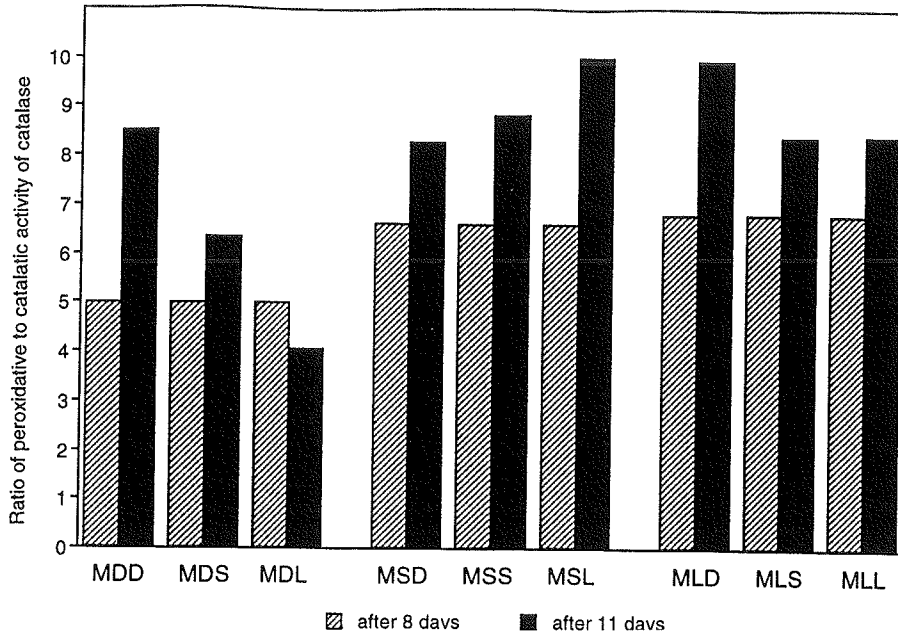

Figure 6. Ratio of peroxidative to catalatic activity of catalase, as defined in Materials and Methods, in leaves of mutant LaPr 86/85 (M) barley seedlings. After a first period of 8 days in the dark (D), shade (S) or light (L), light conditions were changed and the seedlings were grown for another 3 days. For example, "MDL" means mutant, grown first for 8 days in the dark, followed by 3 days in the light.

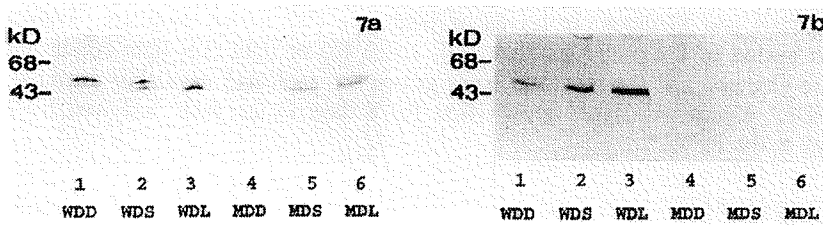

Figure 7. Expression of catalase subunits in leaves of barley seedlings of the wild type and mutant LaPr 86/85, grown first for 8 days in the dark, followed by another 3 days in the dark, shade or light. After SDS-PAGE and Western blotting, the blots were incubated with catalase-specific Mab 39.14 (Fig. 7a) or 39.5 (Fig. 7b). Each lane contained 1.9 μg protein, while the same nomenclature was used as in Figures 5 and 6. Molecular mass markers are indicated on the left.

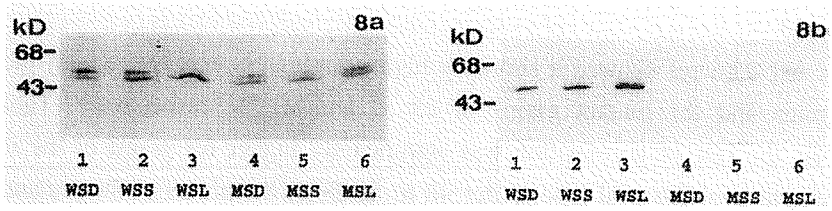

Figure 8. Expression of catalase subunits in leaves of barley seedlings of the wild type and mutant LaPr 86/85, grown first for 8 days in the shade, followed by another 3 days in the dark, shade or light. After SDS-PAGE, and Western blotting, the blots were incubated with catalase-specific Mab 39.14 (Fig. 8a) or 39.5 (Fig. 8b). Each lane contained 1.9 μg protein, while the same nomenclature was used as in Figure 5 and 6. Molecular mass markers are indicated on the left.

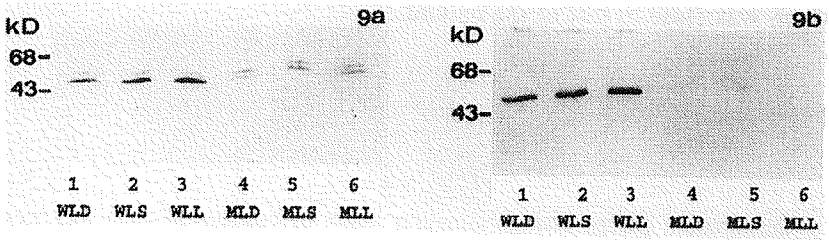

Figure 9. Expression of catalase subunits in leaves of barley seedlings of the wild type and mutant LaPr 86/85, grown first for 8 days in the light, followed by another 3 days in the dark, shade or light. After SDS-PAGE and Western blotting, the blots were incubated with catalase-specific Mab 39.14 (Fig. 9a) or 39.5 (Fig. 9b). Each lane contained 1.9 μg protein, while the same nomenclature was used as in Figure 5 and 6. Molecular mass markers are indicated on the left.

Germination of grains of the catalase-deficient mutant and the wild-type

A significant difference in the rate of germination of grains of the wild type and grains of the catalase-deficient mutant was observed during the germination series. That is, grains of the mutant germinated faster than grains of the wild type. To

quantify these differences in the rate of germination, germination tests were performed at 20 °C, and germination energy and germination index were determined.

These experiments showed a similarly high germination energy for both the wild type grains and the mutant grains of 98 % and 99 %, respectively (Table 1). However, the rate of germination was significantly higher for grains of the mutant than for grains of the wild type. After 48 h, 89 % of the grains of the mutant were germinated, while only 37 % of the grains of the wild type were germinated at this time point. These differences were reflected in a significantly higher germination index for grains of the mutant than for the wild type grains, that is 5.08 and 3.88, respectively (Table 1).

To test if the higher germination rate in the mutant was caused by accumulation of hydrogen peroxide, degradation of which may be impaired in the catalase-deficient mutant, controls were performed in which hydrogen peroxide was added during germination of wild type grains. These tests showed that after addition of exogenous hydrogen peroxide, wild type grains exhibit a germination index of 4.73, which was close to that of the mutant.

Table 1. Germination energy and germination index of catalase-deficient and wild type barley grains, and wild type grains germinated in the presence of hydrogen peroxide. Values are the means of three independent experiments.

	germination energy	**germination index**
catalase-deficient mutant	99 %	5.08
wild type	98 %	3.88
wild type + 2.10^{-3} M H_2O_2	100 %	4.73

DISCUSSION

Using catalase-specific monoclonal antibodies we have shown that the expression levels of the 53 and 57 kD catalase subunits in leaves of young barley seedlings are reflected in the ratio of peroxidative to catalatic activity of catalase, and that their expression is differentially regulated by light. To our knowledge, no work has been reported sofar showing data with respect to the regulation of expression of EP-CAT and T-CAT forms of catalase at the protein level.

Expression of the 57 kD subunit was high during early germination (Fig. 2), or under conditions which repress photorespiration, that is when seedlings were grown in low light (Figs 7 and 8). Under these conditions an enhanced ratio of peroxidative to catalatic activity of catalase was detected. On the other hand, high expression levels of the 53 kD subunit of catalase were linked to normal peroxidative activity of catalase. Expression of the 53 kD subunit was shown to be induced by light, since high levels of the 53 kD subunits were found from day 6 of germination (Fig. 2), or when seedlings were transferred from the dark or shade into the light (Figs 7 and 8). These results nicely complement the data presented by Acevedo *et al.* (1996) and Havir and McHale (1989b), who showed that in barley activity of EP-CAT and T-CAT are regulated by light conditions chosen, and that EP-CAT and T-CAT are encoded by *Cat1* and *Cat2*, respectively. Acevedo *et al.* (1996) showed that etiolated seedlings contain high levels of *Cat1* mRNA, which drop to lower levels when the seedlings were exposed to light.

Our results show that expression of a 57 kD subunit of catalase, which is linked to a high ratio of peroxidative to catalatic activity of catalase, is regulated similarly as *Cat1,* which indicates that this 57 kD subunit is the *Cat1* gene product. Furthermore, regulation of the 53 kD subunit, which is linked to relatively low levels of peroxidative activity, corresponds to regulation of the *Cat2* gene expression, which gene was reported to encode the T-CAT protein (Acevedo *et al.*, 1996). Therefore, we suggest that the 53 kD subunit is the *Cat2* gene product.

It should be emphasized that absolute levels of catalatic activity are much higher than those of peroxidative activity. For instance, after 8 days of germination in leaves of wild type seedlings, which were grown in the dark, catalatic activity was about 170-fold higher than peroxidative activity, while in the light catalatic activity was 2450-fold higher.

Differences between light and dark probably reflect the need to decompose H_2O_2, which is produced at larger concentrations when photorespiration is high. These data are in agreement with results found by Havir and McHale (1989b), who after chromatofocusing of extracts of barley leaves, estimated that EP-CAT constitutes 5 to 10 % of the total catalase activity.

In contrast to the situation in the wild type, expression of the 53 and 57 kD catalase subunits in the mutant were affected only slightly by light conditions. Both the ratio of peroxidative to catalatic activity (Figs 3 and 6) and the expression levels of the 53 and 57 subunits (Figs 4, and 7,8,9) were more or less the same in the dark, shade or light. This may be explained by the fact that the mutant, which was shown to be deficient for the light-inducible *Cat2* mRNA (Acevedo *et al.*, 1996), can express the light inducible 53 kD subunit only to a low, basal level. Also, compared to the situation in the dark, relatively high levels of the 57 kD subunit could be detected in leaves of the mutant grown in the light, maybe to compensate to a certain extent for the reduced expression of the 53 kD subunit (Fig. 4, lane 1 and 3).

The origin of the reduced levels of the 53 kD subunit has not yet been established. It would be interesting to study if the mutant is defective in regulation of the 53 kD subunit because of a deletion of a light responsive element in the promoter of the *Cat2* gene, or because a transacting factor or a protein in the signal transduction chain is eliminated.

The 53 kD subunit, as described in the present paper, probably is similar to the 55 kD subunit in 6-days old barley leaves, grown in the light, as described by Holtman *et al.* (1993). Thus, in barley seedlings at least 3 different subunits are present. In leaves, a 53 kD and a 57 kD subunit, the first induced and the second repressed by light. For aleurone, two subunits have been described previously (Holtman *et al.*, 1993), namely a 57 kD subunit, which may be the same as the one in leaves, and an additional aleurone-specific 51 kD subunit.

For both tobacco (Havir and McHale, 1987) and barley (Acevedo *et al.*, 1996), it was suggested that the T-CAT form of catalase is involved in photorespiration. The present study confirms this, and shows that in barley the T-CAT form contains a 53 kD subunit. It cannot be excluded that T-CAT forms heterotetramers of the 57 and 53 kD subunits, as was described for leaf peroxisomal catalase in pumpkin (Yamaguchi *et al.*, 1984) and catalase expressed in scutellum of maize (Scandalios,

1994). However, the present study suggests that the expression of the 53 subunit regulates the level of the T-CAT form in barley leaves.

The EP-CAT form was shown to be unrelated to photorespiration (Havir and McHale, 1987). Similarly, in the present study the highest absolute levels of peroxidative catalase activity, as well as an increased ratio of peroxidative to catalatic activity were measured in leaves grown under low light conditions, suggesting a role of the EP-CAT form unrelated to photorespiration. In the germination series (Fig. 2), a high expression of the 57 kD subunit was detected at day 4, after which it decreased again. It is probable that EP-CAT is linked to ß-oxidation of fatty acids, which was shown to occur in senescing and dark-treated leaves of wheat and rice (Pistelli *et al.*, 1991), and during early germination in etiolated leaves of barley (Holtman *et al.*, 1994).

Finally, our experiments showed an increased germination index of the catalase-deficient mutant in comparison to that of the wild type. It should be emphasized that these differences in the rate of germination were observed using the same light conditions, thereby excluding the influence of light as a possible explanation for these differences in germination behaviour between grains of the wild type and those of the catalase-deficient mutant (Roth-Bejerano *et al.*, 1996). These results suggest that as a result of increased levels of hydrogen peroxide, a metabolic pathway is induced, which subsequently triggers germination. Catalase-deficient grains probably accumulate hydrogen peroxide to a higher concentration than wild type grains, and therefore exhibit a higher rate of germination.

In this respect, Hendricks and Taylorson (1975), Puntarulo *et al.* (1988) and Fontaine *et al.* (1994) suggested that dormancy breakage and seed germination were accompanied by stimulation of the pentose phosphate pathway of glucose degradation. It was proposed that increased levels of hydrogen peroxide result in a shift to the oxidized state of the NADPH/$NADP^+$ couple, which then leads to stimulation of the pentose phosphate pathway. This model was illustrated by inhibition of catalase in lettuce an pigweed seeds (Hendricks and Taylorson, 1975) and treatment of barley grains with hydrogen peroxide (Fontaine *et al.*, 1994), which both resulted in dormancy breakage. Our results, showing a higher germination index of the catalase-deficient mutant compared to that of the wild-type are consistent with these studies.

This was further substantiated by determining a higher germination index of the

wild type after addition of exogenous hydrogen peroxide. Since plant membranes were shown to be highly permeable to hydrogen peroxide (Stadelmann, 1969), it is likely that addition of hydrogen peroxide to wild type grains results in increased levels of internal hydrogen peroxide, thus mimicking the situation in the mutant.

In conclusion, it appears that activity levels of catalase, which was shown to be an important hydrogen peroxide consuming enzyme in germinating soybean seeds (Puntarulo *et al.*, 1988), may play a crucial role in seed germination.

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Chapter 5

Differential expression of lipoxygenase isoenzymes in embryos of germinating barley

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Differential expression of lipoxygenase isoenzymes in embryos of germinating barley

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ABSTRACT

Expression of lipoxygenase was studied in barley (*Hordeum distichum* L.) embryos during germination. Total lipoxygenase activity was high in quiescent grains, dropped during the 1st day of germination, and subsequently increased to a level similar to that in quiescent grains.

The contribution of two isoenzymes, lipoxygenases 1 (LOX-1) and 2 (LOX-2), was studied at the activity, protein and mRNA levels. Activity ratios of the two isoforms were determined via the ratio of 9- and 13-hydroperoxides, which are formed from linoleic acid. Isoenzyme protein levels were determined using specific monoclonal antibodies. mRNA levels were studied using the specific cDNA probes LoxA and LoxC, which correspond to LOX-1 and LOX-2 respectively.

The major difference in temporal expression of LOX-1 and LOX-2 was observed in quiescent grains. At this stage, LOX-1 contributed almost exclusively to total lipoxygenase activity. LOX-2 activity rapidly increased until day 2 of germination. From this timepoint onward, LOX-1 and LOX-2 showed similar patterns at both activity and protein levels. The tissue distribution of the two isoenzymes in the germinating embryo was closely similar, with the highest expression levels in leaves and roots.

The levels of LOX-1 and LOX-2 may be regulated mainly pretranslationally, as suggested by the similarity of the protein and mRNA patterns corresponding to the two isoforms.

Abbreviations: 9-HPOD, 9-hydroperoxy-10*E*,12*Z* octadecadienoic acid; 13-HPOD,

13-hydroperoxy-9*Z*,11*E* octadienoic acid; Lox/lox, lipoxygenase; Mab, monoclonal antibodies

INTRODUCTION

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a class of non-heme iron-containing dioxygenases that catalyze the oxygenation of poly-unsaturated fatty acids with a 1,4-*cis,cis*-pentadiene structure to form conjugated diene hydroperoxides. The enzyme is widely distributed both in plants (reviewed by Gardner 1991; Siedow, 1991; Vick, 1993) and in animals (reviewed by Schewe *et al.*, 1986; Yamamoto, 1991).

Despite extensive studies, the physiological role of lipoxygenases in plants is poorly understood. It has been suggested that lipoxygenase is involved in plant growth and development (Hildebrand *et al.*, 1991; Siedow, 1991). Highest levels of lipoxygenase activity were found in rapidly growing tissues, suggesting a correlation between lipoxygenase levels and the rate of cell elongation. Furthermore, lipoxygenase may play a role in senescence, wounding and infection, and pest resistance (Gardner, 1991; Siedow, 1991; Vick, 1993). In these processes, the relatively reactive products of the lipoxygenase reaction may cause the observed membrane damage. Furthermore, lipoxygenase may be involved in the biosynthesis of regulatory molecules. For instance, traumatin or "wound hormone", which is formed via the lipoxygenase pathway from the 13-hydroperoxide of linolenic acid, may be involved in the responses of the plant to wounding (Gardner, 1991; Siedow, 1991; Vick, 1993). Another metabolite originating from the lipoxygenase-mediated oxidation of linoleic acid, jasmonic acid, is suggested to cause, among other effects, inhibition of growth, abscission, senescence, and responses to plant wounding and pathogen attack (Gardner, 1991; Creelman *et al.*, 1992; Vick, 1993; Bell *et al.*, 1995). Additionally, lipoxygenase may play a role in the mobilization of lipid reserves (Feussner and Kindl, 1992), and soybean leaf lipoxygenase has been suggested to function as a vegetative storage protein for temporary storage of nitrogen (Tranbarger *et al.*, 1991).

For barley (*Hordeum distichum* L.), two lipoxygenase isoenzymes have been described: LOX-1, which exists in the quiescent as well as in the germinating grain (Yabuuchi, 1976; Baxter, 1982; van Aarle *et al.*, 1991; Doderer *et al.*, 1992; Yang *et al.*, 1993; Hugues *et al.*, 1994), and LOX-2, which generally appears only after germination (Yabuuchi, 1976; Baxter, 1982; Doderer *et al.*, 1992; Yang *et al.*, 1993; Hugues *et al.*, 1994). The two isoenzymes clearly differ in properties. For example, LOX-1 mainly produces the 9-hydroperoxide from linoleic acid, whereas LOX-2 forms primarily the 13-hydroperoxide from this fatty acid (van Aarle *et al.*, 1991; Doderer *et al.*, 1992; Yang *et al.*, 1993; Hugues *et al.*, 1994). This demonstrates that each of the two isoenzymes yields a different subset of lipoxygenase pathway end products and possibly fulfills a distinct physiological role in the germinating barley kernel. However, what these roles could be is unclear at present.

More insight may be obtained by studying the behaviour of the two isoenzymes in embryos of germinating barley. Until now, such information has been rather scarce and has been based solely on activity measurements after separation of both isoenzymes by column chromatography (Yang *et al.*, 1993). Therefore, we have performed a detailed study of the temporal and spatial expression of LOX-1 and LOX-2 during germination of barley at the activity, protein, and mRNA level.

MATERIALS AND METHODS

Plant materials. Barley grains (*Hordeum distichum* L., cv. Caruso, harvest 1992) were germinated between two times three layers of moist filter paper in a plastic plant propagator (33 x 22 cm) at 25 °C in the dark for 9 days. Each day, germinating barley grains were collected and dissected to obtain embryos. From 2 days after germination, embryos were divided into scutella, roots and etiolated leaves including coleoptiles (henceforth simply called "leaves").

Preparation of crude extracts. All steps were performed at 0-4 °C. Crude extracts of (parts of) embryos were prepared by homogenizing 10 grain parts in 20

mM Tris-HCl buffer (pH 7.5) containing 0.5 mM PMSF and 2 mM NaN_3. For extraction, the ratio of buffer (mL) to fresh weight (g) was 10:1, with a minimum of 3 mL. After homogenization using an Ultra-turrax (Janke & Kunkel, Staufen, Germany)(five pulses at 24,000 rpm of 30 s each, with 30 s intervals) the resulting suspension was centrifuged for 5 min at 3,400g. Subsequently the supernatant was centrifuged for 20 min at 15,800g. The supernatant was assayed for lipoxygenase activity immediately and stored at -20 °C for further use.

Enzyme assay and protein determination. Lipoxygenase activity was measured by a spectrophotometric assay as described by Doderer *et al.* (1992). The activity was expressed as μmol hydroperoxide formed per min (unit) at 25 °C per 10 (parts of) embryos using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay, using BSA as a standard (Smith *et al.*, 1985).

Northern blotting. For each sample 10 embryos were dissected from harvested grains, frozen immediately in liquid nitrogen, and stored at -80 °C. RNA was isolated according to Slater *et al.* (1984). From each sample, 7.5 μg of total RNA was denatured by glyoxal/DMSO and separated on a 0.8% agarose gel according to Maniatis *et al.* (1982). Northern blotting was performed using nylon membranes (GeneScreen Plus, DU Pont) according to the manufacturer's instructions. Hybridization of the blots was performed with cDNA probes coding for one of the three barley lipoxygenase cDNAs and containing 142, 227 and 235 nucleotides of the 3' untranslated part of LoxA, LoxB, and LoxC respectively, as determined by J.R. van Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp and B.E. Valk (unpublished data). Nucleotide sequences of LoxA, LoxB and LoxC are available in the EMBL, Genbank, and DNA Data Base of Japan nucleotide sequence databases under accession numbers L35931 (LoxA), L37359 (LoxB) and L37358 (LoxC). The loxA probe encompassed nucleotide positions 2659 to 2801, the loxB probe encompassed positions 1028 to 1255, and the loxC probe encompassed positions 1743 to 1978. One hundred nanograms of each cDNA probe were labelled with a random-priming kit (Pharmacia Biotech, Uppsala, Sweden) in the presence of [α-^{32}P]dCTP and [α-^{32}P]dATP and hybridized with an activity of 10^5 cpm/mL of hybridization mixture.

As a control for equal labelling of the probes dot blots with 5 pg cDNA of LoxA, LoxB, and LoxC were hybridized in the same experiment. The blots were rehybridized with a rRNA probe as a check for equal loading of the lanes.

Identification of 9- and 13-hydroperoxides of linoleic acid.

Crude extracts (100 μL) of whole embryos, prepared as described above, were incubated in 10 mL of 100 mM sodium phosphate buffer (pH 6.5) in the presence of linoleic acid (200 μM) for 40 min with agitation at room temperature. The lipoxygenase reaction was stopped by lowering the pH to 3.5, and 5 μg of prostaglandin B2 were added to the incubation medium as an internal standard. As a control for auto-oxidation, 100 μL of buffer only were incubated with linoleic acid in the same experiment. The products of the lipoxygenase reaction were purified using an octadecyl solid-phase column. The column was washed with 3 column volumes of ice-cold methanol and 2 column volumes of ice-cold water. The incubation medium was then passed through the column followed by washing with 2 volumes of water. Hydroperoxides were eluted with 2 mL of cold methanol.

The products were analyzed by reverse-phase HPLC as described by van Aarle *et al.* (1991) using a ChromSpher C_{18} column (5 μM, 4.6 x 250 mm, Chrompack [Raritan, NJ]), a UV detector (L-4000, Merck-Hitachi, Tokyo, Japan) set at 234 nm, and an integrator (D-2500, Merck-Hitachi). The isocratic solvent was tetrahydrofuran:methanol:water:acetic acid (25:30:44.9:0.1, v/v) adjusted to pH 5.5 with ammonia, and was delivered at a flow rate of 0.5 mL min^{-1}. The pump used was an Intelligent Inert Pump (L-6210, Merck-Hitachi).

Generation of Mab. Female Balb/C mice were immunized intraperitoneally with 30 μg of antigen (highly purified LOX-1 or LOX-2 isolated from germinated barley embryos as described by Doderer *et al.* [1992], and kindly supplied by them) in 200 μL of an emulsion consisting of equal volumes of PBS and complete Freund's adjuvant. With time intervals of 3 weeks, two successive boosters were given with 30 μg of antigen each. Three days after the last booster injection, the spleen was removed and the lymphocytes were used for fusion. For this cell fusion, the mouse myeloma cell line NS-1 was used. The myeloma cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL, catalog no. 42430-025) supplemented with glutamine (2 mM), penicillin (10 units mL^{-1}), streptomycin (10 μg mL^{-1}),

kanamycin (50 μg mL^{-1}), 5x10^{-5} M ß-mercaptoethanol and 10% (v/v) of heat-inactivated bovine calf serum. PEG-induced cell fusion was performed as described by van Duijn *et al.* (1989). Following fusion, the cell population was resuspended in a selective medium (containing 0.1 mM hypoxanthine and 5.8 μM azaserine) in which only hybrid cells can survive, and was distributed over the wells of 96-wells tissue culture plates. About 10 days after this fusion, the antibody production of the hybridomas was tested using an ELISA system as described by de Boer *et al.* (1988). Next, the anti-lipoxygenase-producing hybridoma cells were diluted and seeded over another 96-well tissue culture plate with a cellular density of approximately one cell per well. This so-called "subcloning procedure" was repeated four times with intervals of two weeks. At this stage the cell lines were considered to be monoclonal. To obtain sufficient amounts of Mab, the cloned cell lines were cultured in protein-free hybridoma medium (Gibco).

Lipoxygenase isoenzyme specificity of the Mab was determined using polyacrylamide gel electrophoresis and Western blotting analysis. The Mab secreted by the hybridoma cells were purified from culture medium using protein G affinity chromatography.

Gel electrophoresis and Western blotting. SDS-PAGE was performed on 12.5 % homogeneous gels using the Multiphor II System (Pharmacia Biotech, Uppsala, Sweden).

After electrophoresis, proteins were transferred onto nitrocellulose membrane by semidry blotting, essentially as described by Towbin *et al.* (1979) and Kyhse-Andersen (1984). After blotting, the nitrocellulose membranes were soaked in PBS containing 0.05% Tween 20 plus 1% BSA for 30 min, followed by incubation overnight with LOX-1 or LOX-2-specific Mab (Mab 33.3 and 4.2, respectively), diluted in the same buffer. Subsequently, the membranes were washed with PBS containing 0.05% Tween 20 and incubated for 1 h with goat anti-mouse IgG antibodies conjugated to alkaline phosphatase. Finally, the bound alkaline phosphatase was visualized by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as the substrate. Color development was stopped by washing the nitrocellulose membranes with distilled water.

RESULTS

Temporal expression of lipoxygenase in embryos of germinating barley

Lipoxygenase activity

Activities of lipoxygenase were measured in embryos during germination and expressed as total activity per 10 embryos. Total lipoxygenase activity showed a sharp decrease during the 1st day of germination (Fig. 1). Subsequently, activity gradually increased reaching a maximal value at day 7, that was comparable with the activity in the quiescent grain. Nine days after germination, activity decreased again. The activities of lipoxygenase in the different parts of the embryo will be described below.

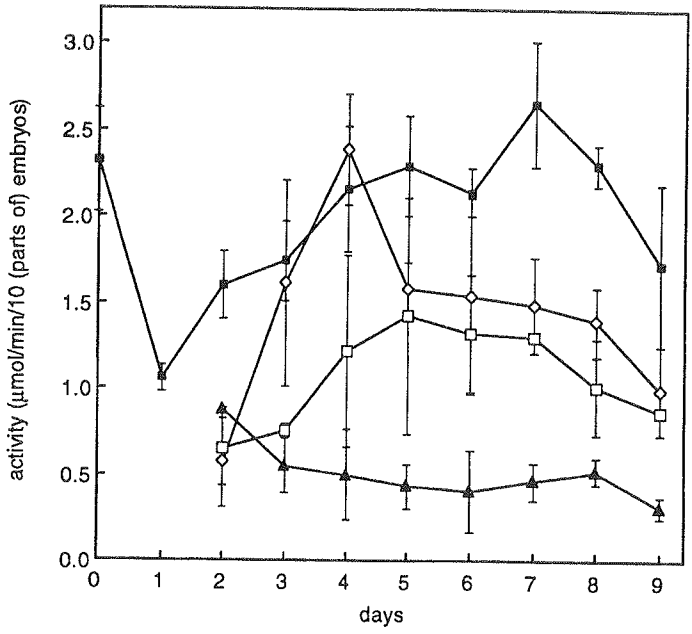


Figure 1. Time course of lipoxygenase activity in total embryo (■) scutellum (▲), leaf (◇), and root (□) of dissected barley embryos upon germination. Activity is expressed as µmol hydroperoxides formed per min^{-1} per 10 (parts of) embryos. Values are the means ± SE of three independent experiments.

To study the contribution of LOX-1 and LOX-2 isoenzymes to total activity, we have determined the ratio of 9- and 13-hydroperoxides of linoleic acid formed by extracts of barley embryos. The ratio of 9- to 13-HPOD formed is an indication of the contribution of LOX-1 and LOX-2, respectively, to total activity during germination. Linoleic acid was used as a substrate in these experiments because it is the most abundant fatty acid in barley (Morrison, 1978) and has been shown to be the preferred substrate for both isoenzymes (Yabuuchi, 1976; Doderer *et al.*, 1992; Yang *et al.*, 1993).

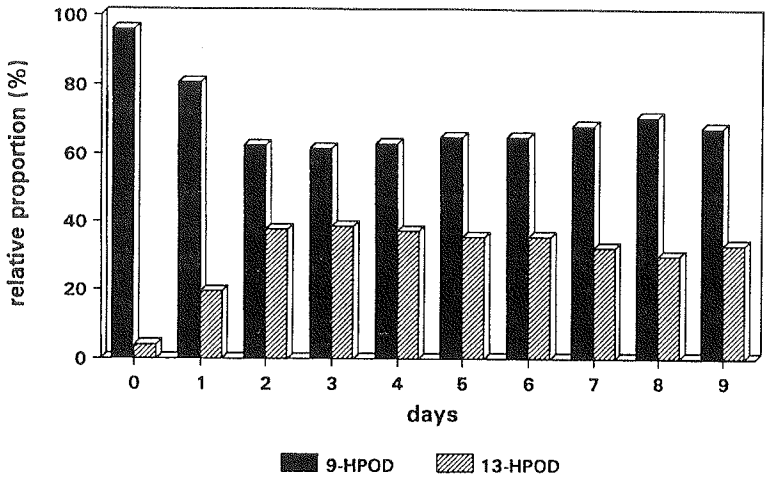


Figure 2. Relative proportion of 9- and 13-HPOD formed from linoleic acid by extracts from embryos from germinating barley.

Figure 2 shows that upon germination, the ratio of 9- to 13-HPOD decreased from 96:4 in embryos from quiescent grains to 62:38 in embryos from 2-days-old seedlings, remaining more or less constant after this time point. These data suggest that the contribution of LOX-1 to total activity decreased during the first days of germination to about 65%, whereas relative LOX-2 activity increased over this time. After 2 days of germination, their relative contributions remained constant.

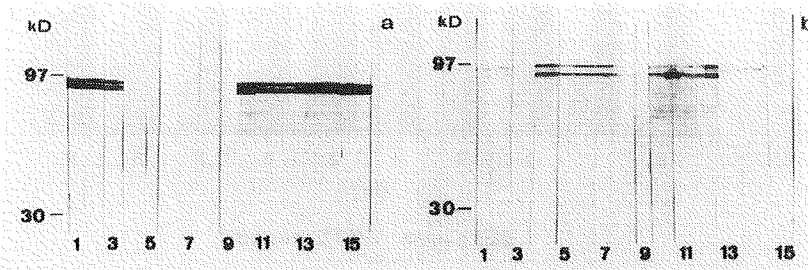
Generation of Mab

The ratio of products formed by lipoxygenase provides an indication of the

relative, but not of the absolute content of LOX-1 and LOX-2 in embryo extracts. One of the ways to study the absolute level of the two isoenzymes is to determine the LOX-1 and LOX-2 protein levels with monospecific antibodies.

After repeated screening in ELISAs and subcloning of the hybridoma cells, six anti-lipoxygenase Mab-producing hybridomas were obtained. Of these six types of Mab, three (2G4, 33.3 and 5D2) showed a pronounced specificity toward barley LOX-1 as determined by ELISA (data not shown). Two Mab (MAbs 4.2 and 5.8) exclusively recognized LOX-2 in ELISA, and one Mab (4.9) recognized both lipoxygenase isoenzymes (data not shown).

These six Mab were purified and further tested with respect to specificity in western blotting experiments. For these immunoblotting experiments, various antibody concentrations were used. Figure 3 shows the reactivity of five different purified Mab with LOX-1 and LOX-2 on western blots.



Lane	Mab	Dilution factor	Lane	Mab	Dilution factor
1	33.3	40	9	5.8	50
2	33.3	100	10	4.9	10
3	33.3	1000	11	4.9	100
4	4.2	10	12	4.9	1000
5	4.2	100	13	5D2	10
6	4.2	50	14	5D2	100
7	5.8	10	15	5D2	1000
8	5.8	100			

Figure 3. Western blotting analysis showing the reactivity of five different purified Mab with LOX-1 (a) and LOX-2 (b). The various Mab were diluted as indicated in the table below the blots.

MAb 33.3 showed exclusive specificity toward LOX-1 at higher dilutions (lane 3), whereas for Mab 4.2 and 5.8 an opposite specificity was observed (lanes 4-9). In agreement with the data obtained from ELISA experiments, Mab 4.9 showed a pronounced reactivity with both LOX-1 and LOX-2 (Fig. 3, a and b, lanes 10-12). Mab 5D2 showed a minor cross-reactivity with LOX-2 at high antibody concentrations. Dilution of this antibody by a factor of 1000 resulted in a complete loss of signal on the western blot of LOX-2, whereas at this concentration the antibody showed a very strong reactivity with LOX-1 (Fig. 3a, lane 15). Finally, the exclusive reactivity of Mab 2G4 toward LOX-1, as observed in ELISA, was confirmed in western blotting experiments (data not shown).

The antibodies recognized a double or even a triple band of proteins with approximate molecular masses of about 90 kD. In the purified LOX-1 and LOX-2 preparations used for western blotting experiments, the polypeptides with lower molecular mass have been suggested to be degradation products of the largest protein (Doderer *et al.*, 1992).

Lipoxygenase isoenzyme protein levels

Levels of LOX-1 and LOX-2 protein in embryos of germinating barley were determined by western blotting experiments with use of the specific Mab 33.3 (reacting with LOX-1) and 4.2 (reacting with LOX-2), respectively, generated and characterized as described above.

Figure 4a shows that LOX-1 was already present in embryos of quiescent grains. On the 1st day of germination, the level of LOX-1 protein in embryos showed a decrease. At days 4 and 5, clearly higher amounts of LOX-1 protein were detected, after which levels decreased again.

Levels of LOX-2 protein are relatively low in embryos of quiescent grain. The increase in LOX-2 protein detected during germination, resulting in highest levels from days 2 to 5 (Fig. 5a), is in agreement with the increasing contribution of LOX-2 to total lipoxygenase activity, as shown in Figure 2. After day 5, LOX-2 protein levels decreased.

These results demonstrate that the decrease in total lipoxygenase activity observed at day 1 of germination was a result of the decreased level of immunoreactive LOX-1 protein in the embryo extracts. The subsequent activity

increase was paralleled by increased protein levels of both isoenzymes. Finally, during the last phase of germination, the decrease in total lipoxygenase activity appeared to be preceded by a decrease in both LOX-1 and LOX-2 proteins.

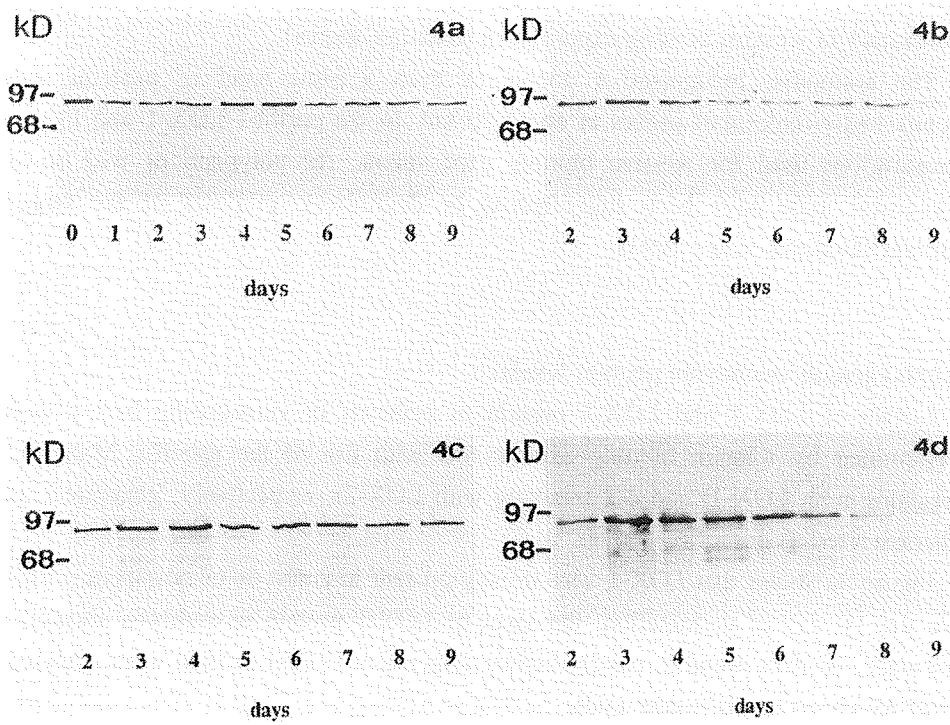


Figure 4. Patterns of LOX-1 protein in total embryo (a), scutellum (b), leaf (c) and root (d) of barley in the course of germination as determined by western blotting using the LOX-1 specific Mab 33.3. The same volume content was loaded on the gel, i.e., 0.006 embryos in each lane in Fig. 4a and 0.011 scutella, 0.011 leaves and 0.011 roots in Figs. 4b, 4c and 4d, respectively. Molecular mass markers are indicated on the left.

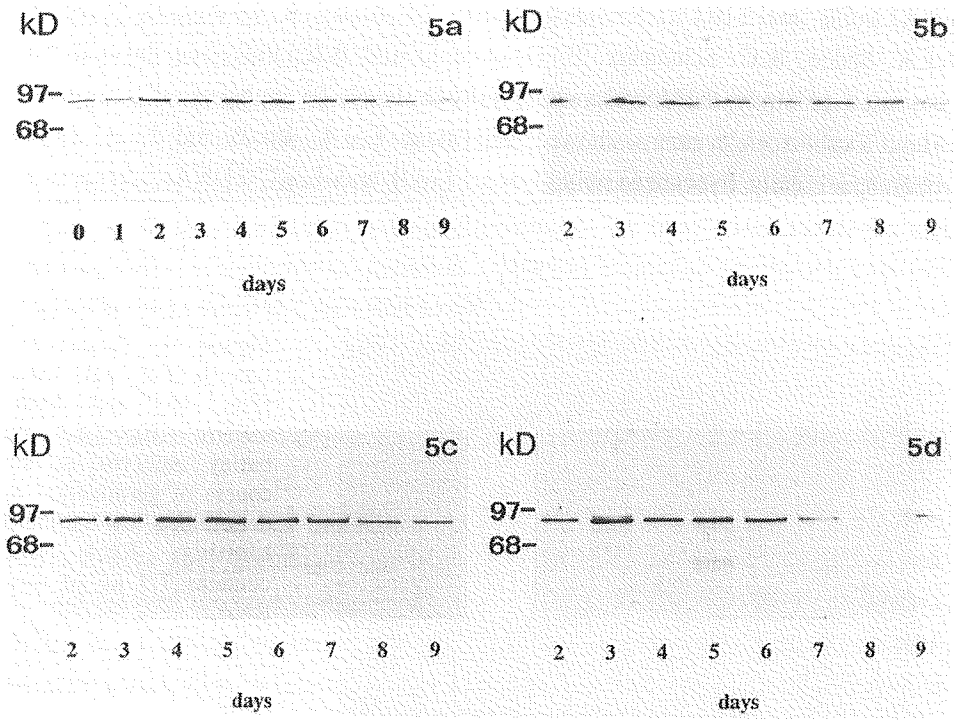


Figure 5. Patterns of LOX-2 protein in the embryo (a), scutellum (b), leaf (c) and root (d) of barley in the course of germination as determined by western blotting using the LOX-2 specific Mab 4.2. Loading of the gel was performed as described for Figure 4. Molecular mass markers are indicated on the left.

Lipoxygenase mRNA levels

A study on the level of the mRNAs corresponding to the two lipoxygenase isoenzymes may indicate at which levels the expression of LOX-1 and LOX-2 are regulated. In barley, three cDNAs, namely LoxA, LoxB, and LoxC have been identified; LoxA and LoxC probably encode LOX-1 and LOX-2 respectively, whereas LoxB encodes an as-yet-unidentified lipoxygenase isoform (J.R. van Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp and B.E. Valk, unpublished data). However, it should be noted that the presence of a third isoenzyme could not be demonstrated in germinating barley (Doderer at al., 1992).

cDNA probes containing 142 to 235 nucleotides of the 3' untranslated region of LoxA, LoxB, and LoxC (J.R. van Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp and B.E. Valk, unpublished data) were used to study the corresponding mRNA levels in embryos of germinating barley. Dotblot experiments showed that these LoxA, LoxB and LoxC cDNA probes specifically hybridized with LoxA, LoxB and LoxC cDNAs, respectively (data not shown).

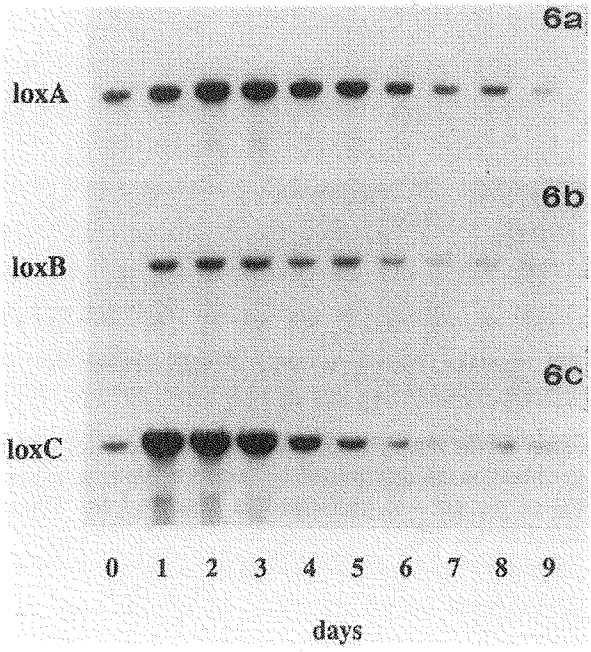


Figure 6. Patterns of Lox A (Fig. 6a), LoxB (Fig. 6b) and LoxC mRNA (Fig. 6c) in barley embryos in the course of germination as determined by northern blotting. Each lane contains 7.5 μg of total RNA.

Figure 6 shows that each of the three probes hybridized with mRNA isolated from embryos from germinating barley. However, there is a remarkable difference in expression patterns of LoxA, LoxB, and LoxC. LoxA mRNA was already present in the quiescent grain (Fig. 6a). Transcripts of LoxA were present throughout germination with highest levels from days 1 to 5. LoxB mRNA could be detected after day 1 only, and, as for LoxA, its highest mRNA levels were found from days 1 to 5 (Fig. 6b). As for LoxB, transcript levels of LoxC rapidly increased during the 1st day of germination (Fig. 6c). The expression of LoxC was most abundant from days 1 to 3, after which it decreased. Increasing levels of

LoxA and LoxC mRNA correlated with increases in LOX-1 and LOX-2 protein during early germination, suggesting that the expression of LOX-1 and LOX-2 are regulated at the pre-translational level.

It should be emphasized that for detection of LoxB mRNA (Fig. 6b) a longer exposure time (i.e. two days) was needed than for detection of LoxA and LoxC mRNA (Figs 6, a and c respectively; exposure time 6 h). Because we have checked for equal labelling of the probes and equal loading of the lanes (data not shown), it seems likely that in embryos of germinating barley, transcript levels of LoxB are less abundant than those of LoxA and LoxC.

Spatial expression of lipoxygenase in embryos of germinating barley

Lipoxygenase activity

From 2 days after germination embryos were divided into scutella, leaves, and roots and assayed for lipoxygenase activity and protein levels of both isoenzymes.

In leaves, a distinct optimum in activity of lipoxygenase was detected at four days after germination, whereas activity in roots showed a broad optimum between days 4 and 7 (Fig. 1). Lipoxygenase activity in scutella was clearly lower than in leaves and roots and stayed more or less constant over time. Division of (total) leaves of 4-days-old seedlings into coleoptile and first leaf revealed that two-thirds of leaf-associated activity resides in the coleoptile (data not shown).

Currently, we have no explanation for the fact that the sum of activities of the different parts of the embryo often exceeds the activity of the whole embryo. For example, protein determinations showed no discrepancy in recovery, and no action of an activity-stimulating or -inhibiting component in any part of the embryo could be detected.

Lipoxygenase isoenzyme protein levels

The presence of LOX-1 and LOX-2 protein was demonstrated in each seedling part investigated and displayed a similar pattern. In leaves, the highest levels are present around day 4 (Fig. 4c and 5c), whereas in roots the highest amount was found from days 3 to 6 (Fig. 4d and 5d). In the scutellum, levels seem to decrease gradually after day 3 (Fig. 4b and 5b). Furthermore, western blotting experiments

showed that, in agreement with activity measurements, the highest protein levels of both LOX-1 and LOX-2 within leaves from 4-days-old seedlings were present in coleoptiles (data not shown).

DISCUSSION

The expression of two lipoxygenase isoenzymes during germination of barley embryos was described at the levels of activity, protein, and mRNA. To our knowledge, this is the first example of such an extensive study in any cereal.

Total lipoxygenase activity in dry barley grains is present at a high level, which rapidly decreases 2-fold during the 1st day of germination and subsequently increases, reaching the same level as dry grain. The rapid drop of activity during the 1st day of germination was observed when activity was expressed both as units/10 embryos and as units/gram tissue (data not shown). An up to 2-fold decrease in activity during early germination was also observed by Lulai *et al.* (1981) and Schwarz and Pyler (1984) and was attributed to lack of oxygen during steeping. This explanation seems unlikely on the basis of the results presented here, since steeping did not form part of the germination procedure. Baxter (1982) and Martel *et al.* (1993) observed that lipoxygenase activity remained constant in the early germination phase. The subsequent increase in activity observed by several authors (Lulai *et al.*, 1981; Baxter, 1982; Schwarz and Pyler, 1984; Martel *et al.*, 1993) varied from 3- to 6-fold, reaching a lipoxygenase activity which was considerably higher than that in the quiescent grain. The differences in lipoxygenase activity patterns might be explained by the use of different barley varieties and germination conditions.

A comparison between LOX-1 and LOX-2 revealed that the only major difference in temporal expression between the two isoenzymes was observed in the quiescent grain. In the dry grain, a high level of LOX-1 was observed, as was evident from hydroperoxide measurements, which showed that lipoxygenase-mediated oxidation of linoleic acid resulted primarily in the production of the LOX-1 product, namely 9-hydroperoxide. In addition, the LOX-1 protein was clearly demonstrable in western blots. LOX-2 protein was also detectable in western blots but, according to the ratio of hydroperoxides formed from linoleic acid, hardly

contributed to the total lipoxygenase activity. The high ratio of 9- to 13-hydroperoxides formed by lipoxygenase from quiescent grains is in agreement with literature values (Yabuuchi, 1976; Baxter, 1982; van Aarle *et al.*, 1991; Yang *et al.*, 1993). LOX-2 was described to be either absent (Yabuuchi, 1976; Yang *et al.*, 1993) or present at a low level (Baxter, 1982; van Aarle *et al.*, 1991), possibly dependent on the barley cultivar used (van Aarle *et al.*, 1991).

From two days after germination onward, LOX-1 and LOX-2 behave similarly. This was evident from the almost constant ratio of hydroperoxides formed by lipoxygenase from 2 to 9 days of germination, which is in agreement with data from Yabuuchi (1976) and Yang *et al.* (1993), and is further supported by the detection of comparable protein patterns for the two isoenzymes. Also in the presence of light, no differences in the expression patterns of LOX-1 and LOX-2 protein in the leaf were observed (data not shown). However, under these circumstances in 4-days-old leaves, total lipoxygenase activity was 2-fold lower than in etiolated leaves (W.L. Holtman, unpublished data). This is in accordance with results described for soybean seedlings, in which a phytochrome-mediated decrease of LOX-1 and LOX-2 activity was found, that was due to transcriptionally regulated gene expression (Maccarrone *et al.*, 1991).

Not only were the temporal expression patterns of LOX-1 and LOX-2 during germination similar, but their distributions in the germinating embryo tissues were also similar, with the highest levels being found in leaves and roots. This result differs from the results of Yang *et al.*, (1993), who observed, based on the ratio of lipoxygenase-derived hydroperoxides formed, that in roots of five-days-old germinated barley, only LOX-2 was present.

Little is known about the regulation of the activity levels of lipoxygenase isoenzymes. During early germination the level of immunoreactive LOX-1 in the extracts decreased and subsequently increased again. This could possibly be due to degradation of lipoxygenase protein. During the later stages of germination the levels of loxA mRNA correspond to those of LOX-1 protein, suggesting a pretranslational regulation. A similar pretranslational regulation was observed for LOX-2.

A third lipoxygenase isoenzyme may be present in germinating barley, as suggested by the expression of a third lipoxygenase mRNA. The protein it encodes probably is present at low levels if at all, since it was not observed during

purification of lipoxygenase isoenzymes from germinating barley (Doderer *et al.*, 1992). This would be in agreement with the low mRNA level for this isoenzyme. It may be present at higher levels, for example during grain development, in the mature plant or under stress conditions (J.R. van Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp and B.E. Valk., unpublished data).

The physiological role of LOX-1 and LOX-2 is unclear at present. The enzymes give rise to different subsets of lipoxygenase products. The major difference in their expression is in the quiescent grain, whereas over the course of germination the two enzymes behave similarly. In soybean, the best-studied plant with respect to lipoxygenase, two classes of lipoxygenase isoenzymes have been described (Kato *et al.*, 1992). Isoforms of the first class, namely L-1, L-2, and L-3, are already present in the quiescent grain and gradually disappear during germination. They have been inferred to play a role in the development of germination capability (Hildebrand *et al.*, 1991) or in plant defense (Kato *et al.*, 1993). LOX-1 from barley is also present in the quiescent grain but also appears to be expressed during germination. Therefore, in the quiescent grain, LOX-1 may function in plant defense or may be involved in the development of germination capability, whereas in the germinating grain, LOX-1 may have a different function. In cotyledons of soybean three isoenzymes of a second class, L-4, L-5, and L-6, appeared after germination, and these may play a role in growth and development (Kato *et al.*, 1992) or, as was indicated for L-4, they may function as a storage protein (Kato *et al.*, 1993). LOX-2 from barley resembles those isoenzymes in this respect, because it shows a similar expression pattern. Further indications regarding the physiological roles of LOX-1 and LOX-2 may be obtained from a study of their location in the tissue and at the subcellular level. The Mab generated as part of this study will form an excellent tool in this respect.

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Chapter 6

Lipoxygenase-2 oxygenates storage lipids in embryos of germinating barley

Submitted

Lipoxygenase-2 oxygenates storage lipids in embryos of germinating barley

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ABSTRACT

Besides the pre-existing lipoxygenase (LOX-1) present in quiescent grains, a new lipoxygenase (LOX-2) is induced in embryos of germinating barley [Holtman, W.L., Van Duijn, G., Sedee, N.J.A. & Douma, A.C. (1996) *Plant Physiol.* 111, 569-576]. The fact that LOX-1 and LOX-2 form different products after incubation with linoleic acid, the 9S-and 13S-hydroperoxides, respectively [Van Aarle, P.G.M., De Barse, M.M.J., Veldink, G.A. & Vliegenthart, J.F.G. (1991) *FEBS Lett.* 280, 159-162; Doderer, A., Kokkelink, I., Van der Veen, S., Valk, B.E., Schram, A.W. & Douma, A.C. (1992) *Biochim. Biophys. Acta* 1120, 97-104], and differ in temporal expression, suggests different physiological functions for both isoenzymes at the onset of germination.

We aimed at obtaining more information about these functions by studying the substrate- and product specificities of both isoenzymes. Analyses of the products formed from linoleic acid confirmed that LOX-1 was a 9-LOX, and LOX-2 was a 13-LOX. With testing more complex substrates, it turned out that LOX-1 as well as LOX-2 were capable of metabolizing esterified fatty acids. K_m values from both isoenzymes for free fatty acids were much lower than for esterified fatty acids (7 to 35-fold for LOX-1 versus 2 to 8-fold for LOX-2, respectively). Interestingly, LOX-1 showed significantly higher K_m values for esterified fatty acids than did LOX-2. This was reflected by analyses of the products formed from dilinolein and trilinolein; LOX-2 formed higher amounts of oxygenated polyunsaturated fatty acids within the esterified lipids than did LOX-1, with a corresponding larger extent of oxygenation.

In order to identify potential endogenous substrates, we analyzed free- and esterified lipids from total lipid extracts from barley after different periods of germination for LOX-derived products. The results indicated that esterified fatty acids were preferentially metabolized by LOX-2 activity. Analysis of the positional specificity within the lipids after alkaline hydrolysis revealed that only 13S-hydroxy derivatives were formed, indicating the *in vivo* action of LOX-2. These data show that LOX-2 is capable of oxygenating storage lipids and suggest that during the onset of germination LOX-2 may be involved in oxygenation of esterified polyunsaturated fatty acids in barley seeds. We suggest that the oxygenation of these lipids preceeds the onset of their catabolism and that the degradation product, 13S-hydroxy-(9Z,11E)-octadecadienoic acid, serves as an endogenous substrate for β-oxidation and therefore as a carbon source for the growing barley embryo.

Keywords: barley; lipoxygenase isoenzymes; oxygenation; storage lipids

Abbreviations: CP-HPLC, chiral phase HPLC; DL, dilinolein; HPOD, hydroperoxy octadecadienoic acid; HOD, hydroxy octadecadienoic acid; LOX, lipoxygenase; RP-HPLC, reversed phase HPLC; SP-HPLC, straight phase HPLC; TAG, triacylglycerol; TL, trilinolein.

Enzymes: Lipoxygenase EC 1.13.11.12; Lipase EC 3.1.1.3

INTRODUCTION

Lipoxygenases (LOX) are enzymes that catalyze the dioxygenation of polyunsaturated fatty acids containing a (1Z,4Z)-pentadiene structure, forming enantiomeric pure S-hydroperoxy fatty acids. They have been shown to be ubiquitous among eukaryotes [1,2]. Multiple isoenzymes of LOX have been described for many plant species, such as soybean, pea, potato, cucumber and rice [3]. Since their expression is developmentally controlled, it seems likely that the individual isoenzymes of LOX have distinct physiological roles. However, till now these roles are poorly understood. Several biological functions were suggested for

LOXs in plants. It has been suggested that LOX plays a role in the biosynthesis of signaling molecules. These molecules may modulate plant environmental and defensive responses under various growth conditions. For example, traumatin, or "wound hormone", may be involved in responses of the plant to wounding [3-5], while jasmonic acid was suggested to mediate responses to pathogen attack and plant wounding, and also may cause abscission, senescence and inhibition of growth [6,7]. Furthermore, LOX may play a role in plant growth and development [8], and may be involved in the mobilization of lipid reserves of oilseeds [9] and in nitrogen partitioning [10].

In germinating barley, two isoenzymes of LOX have been characterized, namely LOX-1 and LOX-2 [11,12,13], while at least three isoenzymes have been described for green leaves upon methyl jasmonate treatment [14]. The fact that LOX-1 and LOX-2 formed different products after incubation with linoleic acid, 9S-and 13S-hydroperoxide, respectively [11,15] and differed in spatial and temporal expression in barley embryos during germination [16,17], suggests that each isoenzyme plays a different role during germination. The aim of the work described in the present paper was testing of this hypothesis. Therefore, we have analyzed products metabolized from complex substrates by LOX-1 and LOX-2 and from lipid extracts at different time points during germination, in order to identify potential endogenous substrates for LOX-1 and LOX-2, as well as products formed by both LOX-isoenzymes. These data, reflecting the *in vivo* situation, will be discussed in relation to the kinetic parameters, which were calculated and the product specificities of LOX-1 and LOX-2. The results open the possibility that in barley, a non-oilseed, a degradation pathway of lipids exists comparable to one in cucumber, where oxygenation of esterified lipids was suggested to occur by a lipid body LOX [18].

MATERIALS AND METHODS

Plant materials. Barley grains (*Hordeum distichum* L. cv Caruso, harvest 1992) were germinated between two times three layers of moist filter paper in a plastic plant propagator (33 x 22 cm) at 25 ^{0}C in the dark. For experiments, embryos

were harvested from 4-days-old seedlings, or otherwise at times indicated.

Purification of LOX-isoenzymes. All steps were performed at 4 °C. Embryos isolated from barley grains, germinated for 4 days at 25 °C in the dark, were used as starting material since at this stage both LOX-isoenzymes were present at relatively high levels [17]. The purification procedure is based on the method described by Yang *et al.* [12]. An amount of 100 embryos (16.3 g fresh weight) was homogenized in 150 mL 10 mM potassium phosphate buffer (pH 6.5) containing 0.5 mM PMSF, 2 mM sodium azide and 0.1 % Triton X-100 (buffer A) using an Ultra-turrax (Janke & Kunkel, Staufen, Germany) (3 pulses at 24,000 rpm of 10 s each, with 30-s intervals). The homogenate was centrifuged at 10,000 g for 30 min. The supernatant was fractionated by addition of solid ammonium sulphate to 20 % saturation. After centrifugation at 10,000 g for 30 min the supernatant was precipitated by addition of ammonium sulphate to 50 % saturation. The pellet obtained after centrifugation (30 min at 10,000 g), which contained most LOX activity, was resuspended in 10 mL buffer A. The sample was applied to a hydroxylapatite column (1.6 x 30 cm). After equilibration of the column with buffer A, a gradient (10-200 mM) of potassium phosphate (pH 6.5) was used for elution, at a flow rate of 1.5 mL min^{-1}. Fractions of 10.5 mL were collected and assayed for LOX activity. Peak fractions were used for further studies.

LOX- and Lipase activity. During the purification procedure LOX activity was measured spectrophotometrically as described by Doderer *et al.* [11]. Activity was expressed as μmoles hydroperoxides formed per minute (Unit) at 25 °C using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}. In substrate specificity studies, when kinetic parameters of LOX-1 and LOX-2 were determined, LOX activity was measured polarographically, by determining the oxygen uptake using a Clark-type electrode (YSI Inc., Yellow Springs, Ohio, USA). Measurements were performed in 200 μL of oxygen-saturated borate buffer (0.2 M boric acid, containing 25 mM HEPES, pH 6.5), which has an oxygen-content of 250 μM at 25 °C. Activity was expressed as μmoles oxygen consumed per minute (Unit). Hydroperoxide-metabolizing enzymes were assayed by monitoring the decrease in absorbance at 234 nm, using hydroperoxy linoleic acid as a substrate [19]. Lipase activity was

assayed in a colorimetric assay using triolein (Sigma) as a substrate, generally as described by MacLeod and White [20]. The free fatty acids released from the substrate, were quantified with use of the NEFA-C kit (WAKO Chemicals Gmbh).

Protein determination. Protein concentrations were determined by the bicinchoninic acid protein assay, using BSA as a standard [21].

Substrate specificity of LOX-1 and LOX-2. To study the specificity of LOX-1 and LOX-2 towards different substrates, LOX activity was measured polarographically (as described above). Trilinolein, dilinolein, methyl linoleate, α-phosphatidylcholine-β-linoleoyl-γ-palmitoyl, linoleic acid, and linolenic acid were used as substrates. Stock-emulsions (24 mM) of the esterified lipids were prepared by emulsification of methyl linoleate, α-phosphatidylcholine-β-linoleoyl-γ-palmitoyl, dilinolein or trilinolein in 2.5 mL Tris-HCl (pH 7.5), containing 15 % (w/v) arabic gum, with an Ultra tip (100 Watt) of a sonifier. Stock solutions (24 mM) of the free fatty acids were prepared by dissolving linoleic or linolenic acid in bidistilled water containing 1 % Tween 20. In a typical assay, 10 μL of the LOX-1 or LOX-2 peak fraction were mixed with borate buffer (0.2 M boric acid, containing 25 mM HEPES, pH 6.5). The reaction was initiated by addition of substrate stock solution.

Product formation by LOX-1 and LOX-2. For determination of LOX-derived products, 200 μL of the LOX-1 and LOX-2 peak fractions (each representing 0.4 Unit of LOX activity), obtained after hydroxylapatite chromatography, were incubated with 125 μL of stock solution of the different substrates (prepared as described in the section above) and 2375 μL borate buffer (see above). Incubations were for 45 min, with agitation, at room temperature. As a control for auto-oxidation, 200 μL of borate buffer only were incubated with the different substrates in the same experiment.

Lipid extraction and sample work-up. After product formation, lipids were extracted for 10 min with agitation in 7.5 mL chloroform:methanol (2:1, v/v) containing 0.11 mM 2,6-Di-tertiary-butyl-4-methylphenol. After centrifugation for 10 min at 1000 g, the organic phase was recovered, the solvent was evaporated,

and the lipids were reconstituted in 100 μL methanol.

Total lipids were extracted from embryos isolated from quiescent barley grains, and from grains germinated for 1 and 4 days. Up to 1.5 g of material was frozen in liquid nitrogen and ground in a Waring blender for 1 min. Generally, the same extraction procedure was used as described above, except that extraction was performed with sonification for 10 min at 25 ^{0}C. After dissolving the lipids in 0.1 mL chloroform, 50 μL were hydrolyzed as described by Feussner *et al.* [18], and were used to determine the oxygenated fatty acids in total lipids, while the other 50 μL were used to determine the amount of oxygenated fatty acids in free fatty acids.

Storage lipids were extracted from lipid bodies. Lipid bodies were isolated from up to 3 g of fresh material by flotation as described before [9] and lipids were extracted as described by Feussner *et al.* [18].

Analytics. HPLC analyses were carried out with a Beckman HPLC system coupled to a diode array detector. The analyses of the free fatty acids and their methyl esters were performed as described before [18].

The analyses of fatty acids within di- and triacylglycerols were performed by RP-HPLC on a Nucleosil C18 column (Macherey & Nagel, KS-system; 250 x 4 mm, 5 μM particle size). A stepwise gradient was used consisting of solvent A (methanol:water:acetic acid; 90:10:0.1, v/v) and solvent B (methanol:acetic acid, 100:0.1, v/v) with the following time program: 0-10 min, 100 % solvent A; 10-30 min, a linear gradient up to 100 % solvent B; 30-80 min, 100 % solvent B. The flow rate used was 1 mL min^{-1}. The absorbances at 234 nm (detection of the conjugated diene system of the hydr(oper)oxy fatty acids) and at 210 nm (detection of the non-oxygenated unsaturated fatty acids) were recorded. Generally, oxygenated compounds were prepared and analyzed after alkaline hydrolysis following the analysis protocol of free oxygenated fatty acids [18], and by co-injections with authentic standards prepared from the corresponding enzymatic reaction products with purified lipid body LOX from cucumber [9]. Compounds separated in the chromatograms were quantified by measuring peak-areas. Calibration curves (5 points measurements) for 13 (S)-hydroxy linoleic acid and linoleic acid were established.

RESULTS

Partial purification of LOX-1 and LOX-2

In order to study the substrate- and product specificity of LOX-isoenzymes from barley embryos, LOX-1 and LOX-2 had to be purified while retaining a relatively high LOX activity. Therefore, a separation method was used, essentially as described by Yang *et al.* [12]. In short, after precipitation with 50 % ammonium sulphate, the LOX-isoenzymes were separated after hydroxylapatite chromatography. Following elution with potassium phosphate, 2 peaks of LOX activity could be detected. The first peak (fraction 15) eluted at 79 mM potassium phosphate, while the second peak (fraction 23) eluted at 102 mM. LOX-forms from fraction 15 and fraction 23 were purified 3.5- and 11-fold, respectively, with respect to specific activity. This is probably an underestimation, which is due to the unstable character of these LOXs.

Product analysis, as described by Feussner *et al.* [18], showed that after incubation with linoleic acid the first peak (fraction 15) formed a ratio of 9- to 13-HPOD of 81:19, demonstrating that this fraction predominantly contains LOX-1 (Table 1). The second peak showed a ratio of 31:69, demonstrating that this fraction primarily contains LOX-2 (Table 1).

Table 1. Analyses of the products from the reactions of fractions 15 and 23 with linoleic acid. Oxygenated linoleic acid derivatives were isolated by RP-HPLC. Positional isomers were given as molar ratios as determined by SP-HPLC. Molar ratios of S and R optical isomers were determined by CP-HPLC.

Fraction	**Positional Isomers** (*13ZE:13EE:9EZ:9EE*)	**Optical Isomers (S:R)**	
		13-ZE-HPOD	*9-EZ-HPOD*
Fraction 15	1:18:59:22	-	97:3
Fraction 23	51:18:11:20	100:0	-

These data are in nice agreement with results described by Yang *et al.* [12] for germinated barley, who found a ratio of 9- to 13-HPOD of 80:20 for LOX-1 and 37:63 for LOX-2 after incubation with linoleic acid. Analysis of the enantiomer composition of the main products of fractions 15 and 23, 9-EZ-HPOD and 13-ZE-HPOD, respectively, revealed that both products were S-isomers (Table 1).

To test separation of the LOX-isoenzymes at the protein level, Western blotting experiments were performed, as described by Holtman *et al.* [17]. These experiments showed that fraction 23 only contained LOX-2, while fraction 15 only contained LOX-1 (data not shown). In fraction 15, besides a distinct band of 90 kD, an approximately 55 kD protein band could be demonstrated, which has been suggested to be a degradation product of LOX [11].

From the results obtained from product analyses and Western blotting experiments, it may be concluded that after hydroxylapatite chromatography the LOX-isoenzymes were almost completely separated and that fractions 15 and 23 contained mainly LOX-1 and LOX-2, respectively. Additionally, in the LOX peak fractions no activity of hydroperoxide-metabolizing enzymes could be detected (data not shown).

Substrate specificity

After separation of the barley embryo LOX isoenzymes by hydroxylapatite chromatography, the fractions containing LOX-1 and LOX-2, respectively, were used to study substrate specificity of both isoenzymes. Relative activities of LOX-1 and LOX-2 for six substrates were determined, namely linoleic acid, linolenic acid, methyl linoleate, dilinolein, α-phosphatidylcholine-β-linoleoyl-γ-palmitoyl, and trilinolein. Using a concentration of 120 μM for each substrate, linoleic acid was shown to be the best substrate for both LOX-isoenzymes. Relative activities of LOX-1 and LOX-2 for the other substrates in comparison to that for linoleic acid are depicted in Table 2.

As can be seen in Table 2, besides linoleic acid, also linolenic acid is a good substrate for both isoenzymes. Interestingly, both isoenzymes can metabolize more complex substrates such as esterified fatty acids, although LOX-2 metabolizes these substrates to a higher extent than does LOX-1. That is, with esterified fatty acids relative activities are 2 to 3-fold higher for LOX-2 than for LOX-1.

Table 2. LOX activities of LOX-1 and LOX-2 using linolenic acid (Le), methyl linoleate (MeLa), dilinolein (DL), trilinolein (TL), and phosphatidylcholine (PL) as substrates, related to activity with linoleic acid (La). Activities were calculated using a concentration of 120 μM for each substrate. Activity with linoleic acid was set at 100 %.

Isoenzymes	Relative Activities				
	Le/La (%)	MeLa/La (%)	DL/La (%)	TL/La (%)	PL/La (%)
LOX-1	70	15	19	7	5
LOX-2	76	32	49	16	7

Next, kinetic parameters were determined for LOX-1 and LOX-2, with use of a range of different substrate concentrations of linoleic acid, linolenic acid, methyl linoleate, dilinolein and trilinolein. Those for phosphatidylcholine could not be determined, because of the very low activities towards this substrate. Using the Lineweaver-Burk equation, in which the reciproke of the reaction velocity is plotted as a function of the reciprocal of the substrate concentration, K_m and V_{max} values were calculated (Table 3). Table 3 shows that the K_m-values from both LOX-1 and LOX-2 for the free fatty acids are much lower than those for the esterified fatty acids (7 to 35-fold for LOX-1 versus 2 to 8-fold for LOX-2). Also, for LOX-1 as well as for LOX-2, highest V_{max} values were calculated when methyl linoleate and dilinolein were used as a substrate. With respect to the free fatty acids, LOX-1 had K_m values slightly lower than those of LOX-2.

However, as was already indicated in Table 2, the most interesting differences between LOX-1 and LOX-2 were found when the esterified fatty acids were used as substrates. LOX-1 shows significant (that is 2 to 3-fold) higher K_m-values for these substrates than does LOX-2. The ratios of K_m versus V_{max} are also shown in Table 3. This value gives an indication about the metabolizing capacity (rate of enzymatic catalysis) of an enzyme for its substrate, if the substrate concentration is

between approximately 0.1 and 10 times the K_m value. Table 3 shows that with respect to LOX-1 this ratio is 5 to 40-fold higher for free fatty acids than for esterified lipids. For LOX-2, on the other hand, this ratio is only 2 to 7-fold higher for free fatty acids than for the esterified lipids. These data suggest that LOX-2 has a relatively higher metabolic capacity for esterified lipids than LOX-1.

Table 3. V_{max}, K_m, and V_{max}/K_m from LOX-1 and LOX-2 for 5 different substrates. K_m was expressed in μM and V_{max} in units/mg protein. Values are the means of two independent experiments.

substrate	LOX-1 V_{max}	LOX-1 K_m	LOX-1 V_{max}/K_m	LOX-2 V_{max}	LOX-2 K_m	LOX-2 V_{max}/K_m
linoleic acid	2.1	26	0.08	4.8	38	0.13
linolenic acid	2.3	50	0.05	7.3	72	0.10
methyl linoleate	5.4	909	0.006	12.7	279	0.04
dilinolein	5.2	537	0.01	18.6	304	0.06
trilinolein	0.95	370	0.002	4.1	174	0.02

To test if LOX activity with esterified fatty acids was not due to oxygenation of free fatty acids, liberated by a putative lipase, commercial lipase (1 μL, 50 units/μL, Sigma) was incubated in addition to the esterified fatty acids in the polarographic activity assay. These tests showed that for all esterified substrates, addition of lipase immediately resulted in a significant increase (2 to 6-fold) of the activity. This can be explained by the fact that by the action of a lipase free fatty acids were liberated, which subsequently were used as preferred substrates. On the

other hand, lipase activity in the LOX-1 peak fraction was very low, and in the LOX-2 fraction even negligible. For instance, using a esterified substrate concentration of 120 μM, the free fatty acids released by the action of lipase from the LOX-1 fraction would contribute to less than one percent of the total amount of substrate. From these results it may be concluded that it is unlikely that LOX activity with esterified lipids is due the oxygenation of free fatty acids, liberated by lipase.

Product formation

Substrate specificity studies, as described above, indicate that both LOX-1 and LOX-2 can oxygenate free fatty acids as well as esterified lipids. Apparently, LOX-2 has a higher affinity for esterified fatty acids than LOX-1. In order to substantiate this difference in specificity between LOX-1 and LOX-2 in more detail, products were analyzed after incubation of LOX-1 and LOX-2 peak fractions with the esterified lipids dilinolein and trilinolein.

Figure 1 shows RP-HPLC chromatograms of the reconstituted lipids after incubation of dilinolein with LOX-1 (Fig. 1A) or LOX-2 peak fraction (Fig. 1B). Whereas at the level of mono-oxygenated dilinolein no clear differences were observed, in the case of the totally oxygenated derivative the situation is completely different. LOX-2 appeared to be capable of oxygenating dilinolein to a drastically higher extent.

In the case of trilinolein as a substrate, the differences were even more clear (Figure 2). Only LOX-2 oxygenated trilinolein in rather large amounts. Interestingly, it converted trilinolein almost completely. The amounts of trilinolein oxygenated by LOX-1 were hardly above the detection limit.

Absorbance spectra at 210 nm, detecting non-oxygenated polyenoic fatty acids, showed that after each incubation, both for dilinolein and trilinolein, significant amounts of the substrate were still available (data not shown). This indicates that it is very unlikely that differences in product formation between LOX-1 and LOX-2 were a result of substrate limitation.

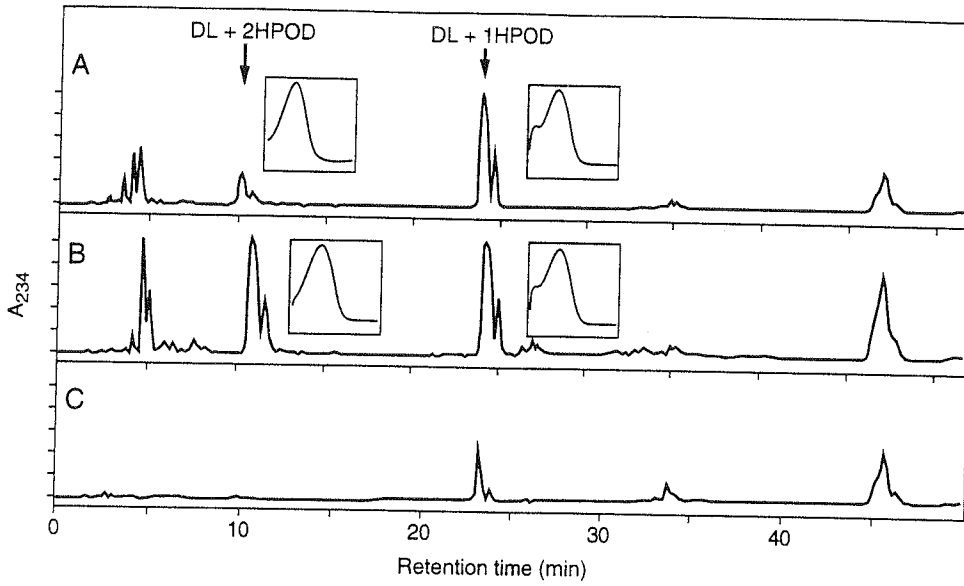

Figure 1. HPLC analysis of esterified products obtained from dilinolein with LOX-1 and LOX-2. LOX peak fractions were incubated with an emulsion of substrate, the remaining lipids were extracted, and analyzed by RP-HPLC as described in the text. The absorbances at 234 nm (detection of the conjugated diene system of the hydroperoxy fatty acids within dilinolein) were recorded (A: LOX-1; B: LOX-2; C: auto-oxidation). The amounts of injected lipids and the sensitivity setting for the UV detector for all traces were the same. The arrows mark the resulting LOX-derived products: DL+2HPOD: dilinolein containing two molecules of HPOD; DL+1HPOD: dilinolein containing one molecule of HPOD. All derivatives were identified by injection of authentic standards. The insets show the corresponding UV spectra recorded from 200-300 nm of the identified derivatives. All spectra showed maximal absorbance at 234 nm.

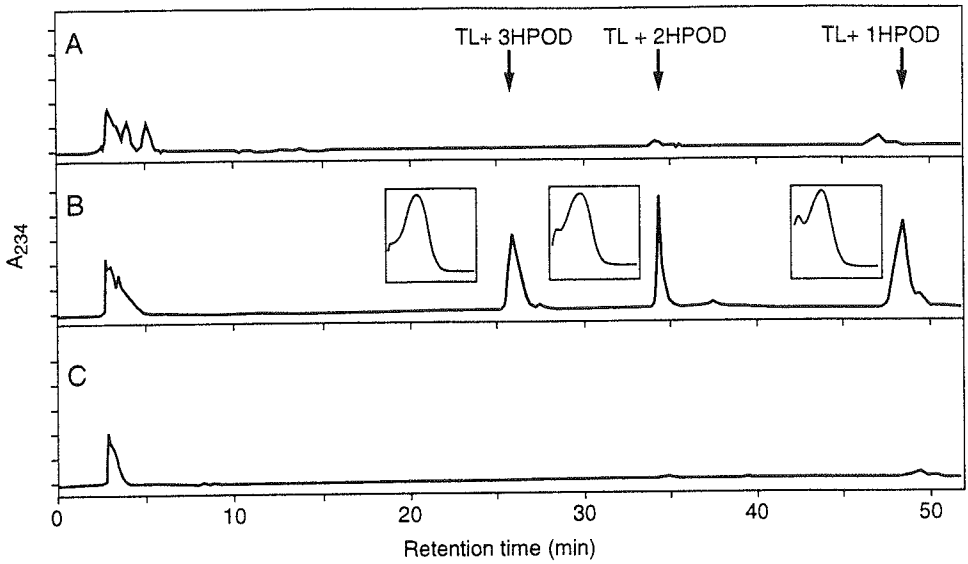

Figure 2. HPLC analysis of esterified products obtained from trilinolein with LOX-1 and LOX-2. LOX peak fractions were incubated with an emulsion of substrate, the remaining lipids were extracted, and analyzed by RP-HPLC as described in the text. The absorbances at 234 nm (detection of the conjugated diene system of the hydroperoxy fatty acids within trilinolein) were recorded (A, LOX-1; B, LOX-2; C, auto-oxidation). The amounts of injected lipids and the sensitivity setting for the UV detector for all traces were the same. The arrows mark the resulting LOX-derived products: TL+3HPOD: trilinolein containing three molecules of HPOD; TL+2HPOD: trilinolein containing two molecules of HPOD; TL+1HPOD: trilinolein containing one molecule of HPOD. All derivatives were identified by injection of authentic standards. The insets show the corresponding UV spectra recorded from 200-300 nm of the identified derivatives. All spectra showed maximal absorbance at 234 nm.

Analyses of lipid extracts for LOX-products during germination

If esterified fatty acids are *in vitro* substrates for LOX-1 and LOX-2, this might be of physiological importance, and the question raises whether this is true for *in vivo* conditions. Additionally, if this process is related to the process of germination, a time-dependent increase in the amounts of oxygenated fatty acids may be expected during the time course of germination. As a suitable measure for the action of LOX we analyzed the amount of HPOD derivatives within lipid extracts in parallel with the S:R ratio of these derivatives. The oxygenated fatty acids within total lipid extracts of embryos have been analyzed after alkaline hydrolysis (Table 4).

Table 4. Analysis of LOX products during the time course of germination. Barley grains were germinated in the dark for various time periods, and the embryos were prepared. Total lipids were extracted and extracts were hydrolyzed under alkaline conditions as described in the text. The amounts of 13- and 9-HOD were determined by SP-HPLC after isolation of all hydroxy fatty acids by RP-HPLC. The S/R ratio was determined by CP-HPLC.

Day	13-HOD nmol/1.5 g	S:R-ratio	9-HOD nmol/1.5 g	S:R-ratio	13:9-ratio
0	88.7	52:48	111.4	47:53	0.80
1	41.8	75:25	15.6	59:41	2.69
4	32.2	91:9	6.7	61:39	4.80

Unexpectedly, the amounts of free 13- and 9-HPOD were below the detection limit. Since hydroperoxy lipids were shown to survive our workup procedure at least in part, a rapid reduction of the corresponding hydroperoxy-derivatives to the more stable hydroxy lipids may be assumed. The analysis at day 0 revealed the largest amounts of hydroxy fatty acids, but analysis on CP-HPLC revealed a racemic mixture of these compounds, suggesting that these products are most likely the result of auto-oxidation. When germination proceeds, the ratio 13:9-HOD increased from 0.8 at day 0 to 4.8 at day 4. And whereas the S:R ratio of 9-HOD

showed only a slight increase, the S:R ratio for 13-HOD increased dramatically during this period, namely from 52:48 to 91:9 (Table 4). These results indicate *in vivo* action of LOX-2 during germination. The fact that these analyses were performed with hydrolyzed lipid extracts suggest that the 13-HOD formed during germination originates from oxygenation of esterified lipids.

To further identify putative endogenous substrates of LOX-2 within lipid extracts, we analyzed non-hydrolyzed extracts of total lipids of day 4 of germination, for oxygenated free fatty acids. In this fraction the amount of 13S-hydroxy-(9Z,11*E*)-octadecadienoic acid, indicative for the action of LOX-2, was below the detection limit (data not shown). Although barley does not belong to oilseed plants we tried to isolate lipid bodies from embryos of day 0, 1, and 4 of germination. From day 0 and day 1, we were able to isolate sufficient amounts for lipid analysis. Interestingly, these data correlate with the findings of the *in vitro* experiments. At day 0 only mono- and di-oxygenated triacylglycerols in a ratio of 4 to 1 could be found, which should be indicative for an unspecific oxygenation (see Table 4). With the induction of LOX-2 at day 1, all three oxygenation products of triacylglycerols were detected in almost equal amounts (data not shown). This could be due to the action of LOX-2.

DISCUSSION

A LOX with specific properties associated to lipid bodies was suggested to be implicated in the onset of the mobilization of storage lipids in cucumber and other oilseeds [18,23]. In the present study we have investigated if a similar degradation pathway of lipids may exist in germinating barley. Therefore we have studied the substrate- and product specificity of two lipoxygenase isoenzymes which have been described for germinating barley, namely LOX-1 and LOX-2 [11,12,13], while additionally endogenous substrates for LOXs and possible products were analyzed in barley grains at different stages of germination.

Substrate specificity studies showed that, although free fatty acids are the preferred substrates for both LOX-1 and LOX-2, esterified fatty acids can be

metabolized as well (Table 2). Interestingly, LOX-2 oxygenates these substrates more easily than does LOX-1. This is in agreement with data presented by Yang *et al.* [12], who showed that esterified lipids could be metabolized by LOX-isoenzymes from germinating barley, in particular by LOX-2. In addition, we have determined kinetic parameters for esterified lipids, besides those for free fatty acids (Table 3). Esterified lipids, mainly triacylglycerols, form the bulk of the storage lipids in barley grains and malt [22]. K_m values of LOX-1 and LOX-2 for free fatty acids were in the same order of magnitude (Table 3), and are in agreement with values reported previously [11,12,15]. However, K_m values of LOX-2 for esterified lipids were significantly lower than those calculated for LOX-1. Furthermore, for LOX-1, the ratio of V_{max}:K_m was 5 to 40-fold higher for free fatty acids than for esterified fatty acids. In comparison, for LOX-2 this ratio was only 1.7 to 6.5-fold higher for the free fatty acids. Taken together, these data indicate that LOX-2 shows *in vitro* more preference for esterified lipids than does LOX-1, and consequently may play a more significant role in degradation of storage lipids than does LOX-1.

A role of LOX-2 in oxygenation of esterified fatty acids was further substantiated by product analyses, after incubation of LOX-1 and LOX-2 with dilinolein and trilinolein. Figures 1 and 2 show that LOX-2 formed higher amounts of oxygenated products, while also oxygenation proceeded to a larger extent than by the action of LOX-1.

These results demonstrated that, at least *in vitro*, esterified fatty acids are metabolized preferentially by LOX-2 in comparison with LOX-1. To investigate whether a similar situation may occur *in vivo*, lipid extracts have been analyzed from barley embryos, isolated from quiescent grains and grains germinated for 1 and 4 days. In lipid extracts of quiescent grains a racemic mixture of hydroxy fatty acids was detected, indicating that the formation of these compounds was probably due to non-enzymatic action. As germination proceeds, the ratio of 13:9-HOD increases significantly (Table 4). Additionally, a clear shift was observed in the enantiomer composition of 13-HOD in favour of the S-isomer. Taken together, these data suggest the *in vivo* action of LOX-2 during germination. Interestingly, the amounts of 9- and 13-HPOD were below the detection limit, suggesting the *in vivo* action of a reductase, which rapidly reduces the more toxic hydroperoxides to their stable hydroxy-derivatives, as was suggested by Feussner *et al.* for cucumber

[23]. The absolute amounts of 13-HOD decrease during germination about 3-fold (Table 4). This is unlike the situation in cucumber [18], where a drastic increase of 13-HOD was detected during germination. It may be that in cucumber and barley different steps control the rate of oxygenation of the esterified lipids and subsequent oxygenation of hydroxy fatty acids. A small, but significant, increase in the S:R ratio of 9-HOD was observed during germination, namely from 47:53 at day 0 to 61:39 at day 4 (Table 4). This increase may be caused by the enzymatic action of LOX-2, since in Table 1 it was already indicated that fraction 23, which was shown to contain LOX-2, could form to a low extent 9-hydroperoxide of linoleic acid, besides the 13-hydroperoxide.

It is not yet clear, which lipid classes represent the endogenous substrates for LOX-2, yielding the high amounts of 13(S)-HOD. In extracts of total lipids of day 4 of germination, the amounts of oxygenated free fatty acids were below the detection limit. It could be likely that within the different lipid classes, the TAGs form the substrate for LOX-2. TAGs account for about 70 % of the total lipid content in barley grains and malt, while about 58 % of the fatty acids are present as linoleic acid [22]. Phospholipids could form a substrate as well. However, they account for only 9 % of the total lipid content in barley grains and malt [22]. It is also very unlikely that the esterified HOD which were detected, originate from oxygenation of free fatty acids. That is, assuming similar turnover numbers of LOX-2 and LOX-1 for linoleic acid [12], and a similar rate of degradation of 9-and 13-HOD, one would suggest that the ratio of 13:9 HOD reflects the relative activities of LOX-2 and LOX-1, respectively, during germination, which at day 4 is about 1:2 [17]. However, our results show that at day 4 the amount of 13(S)-HOD was 5-fold higher than the amount of 9-HOD.

In conclusion, our analyses of lipid extracts from barley embryos and from isolated lipid bodies of this tissue gives a first indication for the action of LOX-2 with respect to storage lipids. LOX-2 may play a role similar to that of another 13-lipoxygenase associated to lipid bodies in cucumber, which was suggested to be involved in the oxygenation of esterified polyunsaturated fatty acids at the onset of germination [18]. Generated lipid hydroperoxides may be further degraded by a recently proposed alternative pathway for ß-oxidation, the reductase pathway [23]. The resulting 13S-hydroxy-(9Z,11E)-octadecadienoic acid may serve as the endogenous substrate for the β-oxidation pathway, which was shown to be active in

the embryos and the aleurone of germinating barley [24], and may serve as source of carbon and energy for the growing embryo.

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Chapter 7

General and summarizing discussion

General and summarizing discussion

Although the lipid content of cereals is relatively low, some parts, such as the embryo and the aleurone layer, may contain a considerable amount of lipids. These lipids, mainly triglycerides, play important roles in determination of physical properties and applications of seed products, flours, starch, proteins (Galliard and Barnes, 1980).

During germination, the lipid content decreases, mainly as a result of hydrolysis of triglycerides and subsequent metabolism of the released fatty acids (Anness, 1984; Holmer *et al.*, 1973). Two metabolic pathways which may contribute to fatty acid oxidation during germination are the ß-oxidation- and lipoxygenase pathway.

The aim of the research described in this thesis was to get a better understanding of the function of these pathways during germination of barley. Since lipoxygenase activity has also been associated with the production of flavor volatiles and bitter tastes (Gardner, 1985), more knowledge about the expression of lipoxygenases in germinating barley may also be of interest for the food industry.

In **Chapter 2**, ß-oxidation was studied in the embryo and aleurone during germination of barley. In order to measure levels of ß-oxidation activity, an overall activity assay, which was used for activity measurements in extracts of human liver (Wanders *et al.*, 1986), was optimized for use in extracts of barley. The main modification was the addition of acyl-CoA synthetase to the incubation medium, which reactivates palmitate, formed by the action of thioesterases, to palmitoyl-CoA. An amount of 300 μg/ml of acyl-CoA synthetase strongly reduced the amount of palmitate formed.

With use of the optimized assay, oxidation of fatty acids by ß-oxidation was followed in the aleurone layer and the embryo of barley during germination. In both the embryo and the aleurone, ß-oxidation activity was already detected in the quiescent grain. During germination, activity levels increased, reaching a distinct optimum in the aleurone at day 4. Highest activity levels of ß-oxidation detected in the aleurone were about 4-fold higher than those detected in the embryo (**Chapter**

2, Figure 3 and 4, respectively).

The relatively lower levels in the embryo may be caused by the presence of another pathway which contributes to fatty acid oxidation in this part of the grain. It was shown that in the embryo lipoxygenase is expressed (**Chapter 5**), while in the aleurone and embryoless grain (A. Doderer, pers. comm. and Yang *et al.*, 1993, respectively) lipoxygenase activity was undetectable or was very low, which was suggested to be partially caused by its incomplete removal from the embryo. It is unlikely that the lower levels of ß-oxidation activity in the embryo are due to a lower amount of substrate available. In another cereal grain, wheat, the amounts of lipids were shown to be even higher in the embryo than in the aleurone (Galliard and Barnes, 1980), while the fatty acid composition in the aleurone and the whole barley grain did not show dramatic differences (Morrison, 1978).

Subcellular fractionation of homogenates of scutella of 4-days germinated grains showed that ß-oxidation was located in the microbodies only. Additionally, in the aleurone, but not in the embryo, enzyme activities of the glyoxylate cycle could be detected. From these data it can be concluded that in the aleurone ß-oxidation is coupled to the glyoxylate cycle, which may lead to the formation of sugars for the benefit of the seedling growth. In barley embryos, ß-oxidation may have a different function. It may be involved in energy generation (Kindl, 1987), or may be part of the general role which peroxisomes were suggested to fulfil in the metabolism of membrane components (Pistelli *et al.*, 1996).

Chapters 3 and **4** deal with catalase, the marker enzyme of microbodies, which degrades the toxic component hydrogen peroxide in water and oxygen. In plants, hydrogen peroxide may be formed as result of two metabolic processes, namely fatty acid ß-oxidation and photorespiration.

Chapter 3 describes the purification and characterization of catalase from young barley leaves. Using the purified enzyme, four cell-lines were generated, which produce catalase-specific monoclonal antibodies (Mabs). These antibodies showed differential recognition of catalase subunits in barley aleurone cells and leaves. So, Mabs from cell-line 39.5 recognizes a 55 kD subunit from barley leaves, and a 51 kD subunit in aleurone cells. Mabs from the other cell-lines, 39.3, 39.10, and 39,14 recognized in addition to the 51 and 55 kD subunits, also a 57 kD subunit in aleurone cells.

The Mabs against catalase proved to be an excellent tool in the electron

microscopical visualization of microbodies: in both aleurone layers and young leaves, protein A gold particles, bound to the Mabs were found over the microbodies only (**Chapter 3**, figure 8). In the aleurone, labelling of catalase was concentrated in the electron dense core of the microbodies. Also in sunflower cotyledons, catalase was after immunogold labelling shown to be concentrated mainly in crystalline inclusions (cores) of peroxisomes (Terberge and Eising, 1995). The physiological function of catalase-containing cores in plant peroxisomes is not yet clear.

Catalase forms with different catalytic properties have been described for maize, tobacco and barley (Havir and McHale, 1989). One form exhibited enhanced peroxidative activity (EP-CAT), another showed "normal" peroxidative activity (T-CAT). Catalase acts peroxidatively at low concentrations ($\leq 10^{-6}$ M) of hydrogen peroxide, which is then degraded with help of a reducing substrate. At high concentrations of hydrogen peroxide, catalase acts catalatically and hydrogen peroxide is degraded directly into water and oxygen (Deisseroth and Dounce, 1970). In maize, (Tsaftaris *et al.*, 1983; Havir and McHale, 1989), tobacco (Havir and McHale, 1989) and barley (Acevedo *et al.*, 1996), both catalytic forms of catalase were shown to be under separate genetic control.

In **Chapter 4**, the regulation of expression and the function of the EP-CAT and T-CAT form in young barley leaves have been studied, thereby using the catalase-specific monoclonal antibodies described in **Chapter 3**. These study showed that an enhanced ratio of peroxidative to catalatic activity of catalase was found in the dark, which was linked to the expression of a 57 kD subunit. Increased levels of a 53 kD subunit were detected after growth in the light, which was linked to normal levels of peroxidative to catalatic activity. These data suggest that EP-CAT and the T-CAT forms are regulated by the expression of the 57 and 53 kD subunit of catalase, respectively.

Since expression of the 53 kD subunit was induced by light, it seems likely that the T-CAT form of barley catalase is involved in photorespiration, which is in agreement with the role of T-CAT described for tobacco (Havir and McHale, 1987).

Most likely the EP-CAT is not related to photorespiration, since highest ratios of peroxidative to catalatic activity were found in the dark. In a germination series (**Chapter 4**, Figure 2), high expression levels of the 57 kD subunit were detected

at day 4, after which the levels decreased again. Therefore, it is probable that the EP-CAT form is related to ß-oxidation, which was demonstrated during early germination in etiolated leaves of barley (**Chapter 2**, Figure 4) and dark-treated leaves of wheat and rice (Pistelli *et al.*, 1991).

One may ask why a plant contains two different catalytic forms of an enzyme, both degrading the same compound, that is hydrogen peroxide. Why, for instance, is the T-CAT form down-regulated in the dark during early germination, and is EP-CAT induced in order to degrade hydrogen peroxide at this developmental stage ? The answer to this question might be given by the fact that a catalase-deficient mutant of barley showed an increased germination index compared to the wild type (**Chapter 4,** Table I). This mutant could only grow under low light conditions or elevated CO_2 levels (Kendall *et al.*, 1983; Parker and Lea, 1983) and it was suggested that the mutant fails to express the T-CAT form (Havir and McHale, 1989; Acevedo *et al.*, 1996).

These data suggest that increased levels of hydrogen peroxide trigger germination. The EP-CAT form may be an excellent tool for the germinating grain to regulate and provide an appropriate hydrogen peroxide concentration. Elevated levels of the T-CAT form during early stages, and consequently lower levels of hydrogen peroxide, might slow down the germination process.

In **Chapter 5**, the expression of two lipoxygenase (LOX) isoenzymes, namely LOX-1 and LOX-2, was studied in embryos of germinating barley at the levels of activity, protein, and mRNA. The main difference in expression of the two isoenzymes was found in quiescent grains. At this stage, LOX-1 almost exclusively contributed to lipoxygenase-activity. This was deduced from product-analysis, showing that after incubation of crude extracts with linoleic acid only the typical LOX-1 product was formed, that is 9-hydroperoxy linoleic acid. Using LOX-specific monoclonal antibodies which were generated, LOX-1 protein was demonstrated to be present in dry grains. Expression of LOX-1 showed a sharp decrease during the first day of germination, both at the levels of activity and protein. After day 1, expression of both LOX-1 and LOX-2 was induced, showing similar patterns both at the levels of activity and protein.

Similar patterns of LOX-1 and LOX-2 protein (**Chapter 5**, Figure 4 and 5) and their corresponding mRNAs, that is loxA and loxC respectively (Figure 6), suggest that expression of LOX-1 and LOX-2 is regulated post-transcriptionally.

It seems likely that LOX-1 exhibits different functions in the quiescent grain and during germination. In the dry grain, lipoxygenase may be involved in the development of germination capability, as was suggested for soybean (Hildebrand *et al.*, 1991), although the exact mechanism is not yet clear. During germination LOX-1 may play a role in nitrogen partitioning (Kato *et al.* 1993), or may be involved in growth and development (Siedow, 1991).

In view of the fatty acid hydroperoxide pathways (Introduction of this thesis), it is likely that LOX-2 derived products in barley are involved in plant responses to stress, pathogen attack and wounding.

Another possible role for barley LOX-2 is described in **Chapter 6**, namely involvement in mobilization of storage lipids, as was suggested for a 13-lipoxygenase from cucumber (Feussner *et al.*, 1995). To test this hypothesis the substrate- and product specificities of barley LOX-1 and LOX-2 were studied. After activity measurements, using LOX-1 and LOX-2 fractions obtained after hydroxylapatite chromatography, kinetic parameters were determined. It was shown that LOX-1 showed higher Km-values for esterified fatty acids than LOX-2. This was confirmed by product analyses which showed that LOX-2 had a higher affinity for dilinolein and trilinolein than LOX-1 (**Chapter 6**, Figure 1). That is, LOX-2 formed higher amounts of oxygenated fatty acids within the esterified lipids than did LOX-1, with a corresponding larger extent of oxygenation. Since the situation *in vitro* not necessarily reflects the situation *in vivo*, the oxygenated fatty acids within total lipids were analyzed from barley embryos isolated from quiescent grains and from grains germinated for 1 and 4 days. These analyses showed that as germination proceeds the ratio of 13-and 9-hydroxy fatty acids (HOD) increased (**Chapter 6**, Table 4). And whereas the S/R ratio for 9-HOD showed only a slight increase, the S/R ratio for 13-HOD increased dramatically from day 0 to 4. These results suggest the *in vivo* action of LOX-2 during germination. Interestingly, the amounts of hydroperoxy fatty acids were below the detection limit, indicating the action of a lipid hydroperoxide reductase, as was suggested for cucumber (Feussner *et al.*, 1997).

It is not yet clear, which lipid classes represent the endogenous substrates for LOX-2, yielding the high amounts of 13(S)-HOD formed during germination. In non-hydrolyzed extracts of total lipids of day 4 the amount of oxygenated free fatty acids were below the detection limit. Most likely, triacylglycerols form the

substrate for LOX-2, since they account for 70% of the total lipid content in barley and malt (Anness, 1983). It is unlikely that phospholipids or free fatty acids form the substrate for the 13-hydroxy fatty acids formed. Phospholipids account for only 9% of the total lipid content, and were shown to be poor substrates for lipoxygenase (**Chapter 6**, Table 2). Furthermore, when free fatty acids would form the substrate for the 13-HOD formed, one would expect a relation between the LOX-2 and LOX-1 activity during germination and the ratio of 13-HOD and 9-HOD, respectively. Such a relation was not observed.

In conclusion, the data described above suggest that LOX-2 is involved in the oxygenation of storage lipids at the onset of germination. The lipid hydroperoxides may be further degraded by a recently proposed pathway, the reductase pathway (Feussner *et al.*, 1997). The resulting hydroxy fatty acids might form the substrate for ß-oxidation.

So, in embryos of germinating barley the lipoxygenase and ß-oxidation pathway may cooperate in the degradation of storage lipids. By the action of lipoxygenase, the lipid body membrane is disrupted (Feussner *et al.*, 1997), and the initial oxygenation of fatty acids within triacylglycerols is facilitated. After the action of a specific lipid hydroperoxide reductase, and a lipid hydroxide lipase the released hydroxy fatty acids form the substrate for ß-oxidation.

The fact that during germination of barley ß-oxidation activity in embryos was significantly lower than in the aleurone (**Chapter 2**), suggests that beside the reductase pathway other fatty acid hydroperoxide degrading pathways are active as well, which will not lead to the formation of substrates for ß-oxidation. Indeed, hydroperoxide lyase, allene oxide synthase, and epoxygenase/peroxygenase activity have been shown for embryos of ungerminated barley (Van Aarle, 1993), while also fatty acid hydroperoxide consuming activity has been detected in extracts of germinated barley (Holtman, unpublished data). Analyses of the secondary products of the lipoxygenase pathway during germination might give more insight into the contribution of the various fatty acid hydoperoxide degrading pathways during germination.

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SAMENVATTING

Een groot deel van het dieet van mens en dier is afkomstig van granen. In het zaad zijn voedselreserves in de vorm van suikers, eiwitten en lipiden opgeslagen. Deze reserves worden gebruikt tijdens de kieming en komen ten goede van de groeiende kiemplant.

In vergelijking met veel andere zaden, zoals b.v. koolzaad en pinda, is het gehalte aan lipiden in granen relatief laag. Toch bevatten enkele delen van het zaad, zoals het embryo en het aleuron, een relatief hoog gehalte aan lipiden. Lipiden kunnen een grote rol spelen bij de fysische eigenschappen en de toepasbaarheid van de zaadprodukten.

Wat betreft produktiehoeveelheden van verschillende graansoorten staat gerst wereldwijd op de vierde plaats. Gerst wordt met name gebruikt voor de produktie van bier en als veevoer. Circa 4.4 % van het drooggewicht van gerst bestaat uit lipiden. Deze bestaan voor 70 % uit triacylglyceriden. Tijdens de kieming wordt circa 30% van de lipiden afgebroken. Na hydrolyse van de triacylglyceriden kunnen de ontstane vetzuren verder worden afgebroken.

In dit proefschrift zijn twee enzymatische routes bestudeerd die tijdens de kieming bijdragen aan de oxidatieve afbraak van vetzuren, namelijk de ß-oxidatie- en de lipoxygenase route. Om een beter begrip te krijgen van de functie van beide routes voor de plant tijdens het kiemproces, werden deze bestudeerd in kiemende gerst.

Tijdens afbraak van vetzuren door ß-oxidatie wordt in vier achtereenvolgende enzymatische stappen een 2C-eenheid van de vetzuurketen (C_n) afgesplitst (Figuur 1 van de inleiding). Eén ß-oxidatie-ronde leidt derhalve tot acetyl-CoA en C_{n-2}. Vetzuur afbraak d.m.v. ß-oxidatie kan leiden tot de generering van energie en de synthese van suikers.

Afbraak van vetzuren door lipoxygenase leidt tot een breed scala van produkten (Figuren 3-6 van de Inleiding), waarvan enkele dienen als signaalstoffen voor de plant.

Aangezien lipoxygenase-activiteit en de als gevolg daarvan ontstane produkten geassocieerd worden met het ontstaan van al dan niet wenselijke smaakstoffen, zal meer kennis van deze route ook relevant zijn voor de voedingsmiddelen-industrie.

In **Hoofdstuk 2** is de activiteit van de ß-oxidatie-enzymen in het embryo en het aleuron van kiemende gerst bestudeerd. Daartoe werd allereerst een al bestaande methode aangepast, waardoor het mogelijk werd om de activiteit van alle bij de ß-oxidatie betrokken enzymen tegelijkertijd in extracten van gerst te meten. De voornaamste aanpassing was toevoeging van het enzym acyl-CoA synthetase, dat de dissociatie van geactiveerd substraat sterk reduceert.

Met behulp van de geoptimaliseerde methode werd de ß-oxidatie-activiteit bepaald in het aleuron en in het embryo. In zowel het embryo als in het aleuron van ongekiemde gerst werd al activiteit gemeten. De activiteit nam echter toe tijdens kieming. De maximale ß-oxidatie activiteit in het aleuron, vier dagen na het begin van de kieming, was circa vier maal hoger dan die in het embryo (**Hoofdstuk 2**, Figuren 3 en 4). Dit kan wellicht verklaard worden door de aanwezigheid van een andere oxidatieve route, die tevens bijdraagt aan vetzuurafbraak in het embryo.

Verder werden organelfracties van een extract van scutellum (een onderdeel van het embryo) geïsoleerd d.m.v. centrifugatie. De scheiding is daarbij gebaseerd op een verschil in soortelijke massa van de verschillende typen organellen. Het bleek dat ß-oxidatie activiteit vooral te meten was in een fractie die microbodies bevatte.

In het aleuron, echter niet in het embryo, werd ook de activiteit van een andere enzymatische route gemeten, nl. de glyoxylaat cyclus. Dit wijst erop dat ß-oxidatie in kiemende gerst in het aleuron in glyoxysomen gelokaliseerd is, een microbody-type waarin ß-oxidatie gekoppeld is aan de glyoxylaat cyclus. Dit suggereert dat vetzuurafbraak door ß-oxidatie in het aleuron zal leiden tot de vorming van suikers ten behoeve van de groeiende kiemplant. Vetafbraak in het aleuron zou ook kunnen leiden tot de vorming van malaat. Deze component zou kunnen zorgen voor verzuring van het endosperm. Als gevolg hiervan kan het aleurone dan al bij lage concentraties van het hormoon gibberellinezuur hydrolytische enzymen uitscheiden, die kunnen dienen voor afbraak van zetmeel. In het embryo, waar geen glyoxylaat cyclus actief is, zal ß-oxidatie een andere functie hebben. Gedacht kan worden aan energieproduktie of aan een rol in het metabolisme van membraan componenten.

In de **hoofdstukken 3** en **4** is het enzym catalase bestudeerd. Dit enzym wordt

beschouwd als merker van microbodies. Het breekt waterstofperoxide af, een voor de cel schadelijk stof.

Waterstofperoxide komt in planten vrij als bijprodukt van twee processen, namelijk bij ß-oxidatie van vetzuren (Figuur 1 in de Inleiding) en bij fotorespiratie, een proces dat plaats vindt in fotosynthetiserende weefsels.

In **Hoofdstuk 3** wordt de zuivering beschreven van het enzym catalase uit blad van zes dagen oude gerst-kiemplanten. Het gezuiverde enzym werd gekarakteriseerd, d.w.z. de molecuul-grootte werd bepaald, alsmede het isoelektrisch punt en de pH waarbij het enzym zijn optimale activiteit vertoont. Vervolgens zijn met behulp van het gezuiverde enzym monoclonale antilichamen-producerende cellijnen gegenereerd, die specifiek catalase herkennen. Van de vier cellijnen herkende er één specifiek een 51 kD catalase subunit in aleuron cellen en een 55 kD subunit in blad. De andere drie cellijnen herkenden behalve de hiervoor genoemde subunits nog een 57 kD catalase subunit in aleuroncellen.

De catalase-specifieke monoclonale antilichamen bleken zeer geschikt voor het zichtbaar maken onder de electronenmicroscoop van microbodies in blad en aleuron. Proteine A goudbolletjes, die koppelden aan de catalase-herkennende monoclonale antilichamen, waren alleen te zien op microbodies (Hoofdstuk 3, Figuur 8).

De monoclonale cellijnen werden vervolgens in **Hoofdstuk 4** gebruikt om in blad van gerst de regulatie van de expressie en de functie van twee katalytisch verschillende vormen van catalase te bestuderen. Catalase kan waterstofperoxide katalatisch afbreken en vormt daarbij zuurstof en water. Het kan waterstofperoxide ook peroxidatief afbreken, daarbij gebruik makend van een reducerend substraat.

In de literatuur zijn voor mais, tabak en gerst catalase-vormen beschreven met verschillende katalytische activiteit, nl. een vorm met verhoogde peroxidatieve activiteit (EP-CAT) en een vorm met "normale" peroxidatieve activiteit (T-CAT). Gesuggereerd wordt dat EP-CAT en T-CAT onder controle staan van verschillende genen en dat licht een rol speelt bij de mate van expressie van beide vormen.

In **Hoofdstuk 4** is beschreven dat bij in het donker groeiende gerst een verhoogde peroxidatieve activiteit van catalase werd gemeten, welke gerelateerd is aan de expressie van een 57 kD catalase subunit. Daarentegen kwam bij groei in het licht juist een 53 kD subunit tot expressie en dit was gerelateerd aan normale peroxidatieve activiteit. Dit wijst erop dat de EP-CAT en T-CAT gecorreleerd zijn

met respectievelijk een 57 en 53 kD catalase subunit. Het feit dat T-CAT tot expressie komt in het licht doet veronderstellen dat dit type catalase het waterstofperoxide afbreekt dat bij fotorespiratie vrij komt.

De EP-CAT vorm komt juist tot expressie in het donker, tijdens het begin van de kieming. Het is waarschijnlijk dat de EP-CAT vorm betrokken is bij de afbraak van waterstofperoxide dat ontstaat bij ß-oxidatie van vetzuren.

Het feit dat een catalase-deficiënte mutant sneller kiemt dan het wild-type, suggereert dat catalase-nivo's tijdens het begin van de kieming van belang zijn voor de kiemsnelheid.

In **Hoofdstuk 5** is een studie beschreven waarin in kiemende gerst de expressie bestudeerd is van twee verschillende vormen (iso-enzymen) van lipoxygenase (LOX), LOX-1 en LOX-2. Lipoxygenase is het enzym dat de eerste stap katalyseert van een oxidatief proces dat leidt tot de afbraak van meervoudig onverzadigde vetzuren. Bij de lipoxygenase-reactie wordt zuurstof ingebouwd in de dubbel onverzadigde binding van deze vetzuren (Figuur 2 van de Inleiding). Het primaire produkt dat hierbij ontstaat, een hydroperoxide van een vetzuur, kan vervolgens enzymatisch en non-enzymatisch verder worden afgebroken.

Vier routes waarlangs de hydroperoxides van vetzuren enzymatisch verder kunnen worden afgebroken, zijn weergegeven in de Figuren 3,4,5 en 6 van de Inleiding. Sommige van de ontstane produkten hebben een belangrijke fysiologische functie voor de plant. Bovendien zijn met name de gevormde aldehyden en ketolen erg vluchtig en kunnen een karakteristieke smaak aan voedsel geven.

Voor lipoxygenase-activiteit in ongekiemde gerst is alleen LOX-1 verantwoordelijk. LOX-2 komt pas in de loop van de kieming tot expressie. Vanaf dag 2 van de kieming vertonen LOX-1 en LOX-2 een vergelijkbaar expressiepatroon op zowel eiwit- als activiteitsnivo (**Hoofdstuk 5**, respectievelijk de Figuren 4 en 5).

Het is waarschijnlijk dat LOX-1 in ongekiemde gerst een andere functie vervult dan in gekiemde gerst. Welke die functie is, is niet geheel duidelijk. In ongekiemde gerst zou LOX-1 een rol kunnen spelen bij het ontwikkelen van de kiemkracht van het zaad. Tijdens de kieming zou LOX-1 een functie kunnen hebben bij de groei en de ontwikkeling van de plant, of bij het opslaan van stikstof in het blad.

LOX-2 is waarschijnlijk betrokken bij reacties van de plant in een situatie van stress, verwonding, of een aanval door een pathogeen.

Een andere mogelijke functie van LOX-2 is beschreven in **Hoofdstuk 6**, nl. de betrokkenheid bij de afbraak van lipiden die in het zaad zijn opgeslagen. Om daarin duidelijkheid te krijgen werden de substraat- en produkt specificiteit van LOX-1 en LOX-2 bestudeerd. Gezuiverde LOX-1- en LOX-2 preparaten werden daartoe geïncubeerd met verschillende soorten lipiden. Uit metingen van lipoxygenase-activiteit bleek dat LOX-1 een lagere affiniteit dan LOX-2 vertoonde voor veresterde vetzuren, zoals dilinoleinezuur en trilinoleinezuur. Dit resultaat werd bevestigd door analyse van de gevormde produkten.

Aangezien deze *in vitro* experimenten niet vanzelfsprekend de situatie in de levende plant weergeven (de *in vivo* situatie), werden daarom tevens lipiden geanalyseerd in extracten van embryo's uit gekiemd en ongekiemd zaad. Duidelijk werd dat naarmate de kieming verder was gevorderd de verhouding van hoeveelheden tussen 13- en 9 hydroxyvetzuren toenam. Het feit dat de aanmaak van 13-derivaten van vetzuren juist door LOX-2 gekatalyseerd wordt (Figuur 2 van de Inleiding) wijst op de *in vivo* activiteit van LOX-2.

De omzetting van hydroperoxiden van vetzuren in hydroxyvetzuren zou kunnen gebeuren via de reductase-route (Figuur 6 van de Inleiding). Welke substraten LOX-2 voor de vorming van deze 13-hydroxyvetzuren gebruikt is niet geheel duidelijk. Waarschijnlijk zijn dat triacylglyceriden die, zoals als eerder gememoreerd, de bulk van de opslaglipiden in het zaad vormen.

Voorgaande resultaten doen veronderstellen dat tijdens kieming LOX-2 betrokken is bij de oxidatieve afbraak van opslaglipiden in het zaad. De gevormde hydroxyvetzuren zouden dan verder afgebroken kunnen worden d.m.v. ß-oxidatie en dat kan leiden tot de vorming van suikers en het ontstaan van energie voor de plant. Het feit tijdens de kieming de ß-oxidatie-activiteit in het embryo significant lager is dan in het aleuron (**Hoofdstuk 2**), wijst erop dat het primaire LOX-2 produkt, nl. 13-hydroperoxide van linolzuur, behalve via de reductase route (Figuur 6), ook via andere routes afgebroken kan worden.

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CURRICULUM VITAE

Wessel Luitje Holtman werd geboren op 8 juni 1962 te Ten Boer. Na het behalen van het VWO-diploma aan het Augustinuscollege te Groningen, in juni 1980, werd in augustus 1980 begonnen met een studie aan het Instituut voor de Opleiding van Leraren Ubbo Emmius in Groningen. In juni 1985 werd examen afgelegd en werd een tweede graads bevoegdheid verkregen in het vak Biologie en een derde graads bevoegdheid in het vak Scheikunde. In 1985 werd begonnen met het doctoraalprogramma Biologie oude stijl aan de Rijksuniversiteit Groningen. Gedurende deze studie werd een hoofdvak gedaan bij de werkgroep microbiële fysiologie (Prof. dr. W. Harder, dr. M. Veenhuis) en een bijvak bij de vakgroep Biochemie (Prof. dr. M. Gruber, dr. G. AB). Het doctoraalexamen biologie werd afgelegd in november 1988, waarbij tevens een eerste graads bevoegdheid werd verkregen tot het geven van onderwijs in het vak Biologie. Vanaf 1 april 1989 is hij in dienst bij de Nederlandse Organisatie voor toegepast-natuurwetenschappelijk onderzoek TNO en is werkzaam bij het Centrum voor Fytotechnologie RUL-TNO, afdeling Plantenbiotechnologie in Leiden.