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Subcellular Distribution of Palmitoyl-CoA Hydrolase Activity in Carrot—An Enzyme Releasing Free Fatty Acids

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ABSTRACT

Palmitoyl-CoA hydrolase releases free fatty acids which are regarded as precursors of metabolites giving off-flavour in unblanched frozen carrot cubes. The subcellular distribution of the enzyme has been studied by differential centrifugation using marker enzymes. Palmitoyl-CoA hydrolase activity was mainly found in the cytosol, but a membrane bound endoplasmatic and mitochondrial localization was also found. The importance of the enzyme during cell disruption is discussed.

INTRODUCTION

Unblanched frozen carrot cubes developed off-flavour and off-taste, characterized as stearin, soap and paraffin, when stored at ~20 °C for 7 months (Baardseth & Slinde, 1983*b*). In plant fatty acid oxidation, free fatty acids may be metabolized through α-oxidation, ω-oxidation or by the action of lipoxygenase. Free fatty acids are formed from complex lipids by the action of lipases or by hydrolysis of activated fatty acids such as fatty-acyl-CoA (Galliard, 1975).

When plant materials are stored or wounded, catabolic reactions predominate and the free fatty acid pool is probably elevated (Baardseth & Slinde, 1983*a*). The rate of free fatty acid oxidation may increase due to disruption of cell compartments, thus mixing enzymes and substrates originally separated by membranes in the intact plant. Lipoxygenase and

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α-oxidation produce aldehydes that give characteristic taste or flavours to, for example, cucumber, but in other cases the compounds may be characterized as off-flavour (Galliard, 1975).

Lipoxygenase activity has not been detected in carrot (Baardseth & Slinde, 1980*a*), but palmitoyl-CoA hydrolase activity (1·54 nmol per milligram of protein per minute) has been found in carrot homogenate (Baardseth & Slinde, 1980*b*) and the importance of this enzyme in off-flavour production has been discussed (Baardseth & Slinde, 1983*b*). The subcellular localization of palmitoyl-CoA hydrolase in plant tissues has not yet been studied, but long-chain acyl-CoA hydrolases, associated with the envelope of spinach and pea chloroplasts, have been characterized (Joyard & Stumpf, 1980; Andrews & Keegstra, 1983). A short-chain acyl-CoA hydrolase has been located in the matrix space of spinach leaf mitochondria (Liedvogel & Stumpf, 1982), and in potato, a carboxylic ester hydrolase was found, mainly in the post-microsomal supernatant (Galliard, 1970; Matsuda & Hirayama, 1979).

The aim of the present work was to study the subcellular distribution of palmitoyl-CoA hydrolase activity in carrot. This was performed by gentle homogenization followed by differential centrifugation, essentially as described by De Duve *et al.* (1955), and marker enzymes were used to identify the subcellular components. The distribution of palmitoyl-CoA hydrolase activity may indicate to what extent a possible production of off-flavour may be generated in sliced or wounded tissue.

MATERIALS AND METHODS

Carrots (*Daucus carota* L., var. Nantes Duke), grown at the Agricultural University of Norway, were harvested at maturity and stored at 0 °C and 95% relative humidity. All chemicals were of analytical grade. Cytochrome *c* was from Sigma Chemical Co., St. Louis, USA, and [1-^{14}C]-palmitoyl-CoA (specific activity, 57 μCi mg^{-1}) was purchased from Amersham International, Amersham, Great Britain.

Subcellular fractionation

The carrots were washed, peeled and coarsely cut. The cut carrots (120 g) were homogenized with either an Ultra-Turrax TP 18/10 or a Robot Vertical Cutter 2, with 120 ml buffer (0·4 mol sucrose per litre, 15 mmol

HEPES per litre, pH 6·3) at 4 °C. After filtration through cheesecloth, the resulting homogenate (H) was fractionated according to the principles of De Duve *et al.* (1955).

The nuclear fraction (N) was sedimented by centrifugation in a Sorvall RC2-B centrifuge with the HB-4 rotor at $\int_0^t \text{rpm}^2\,dt = 8{\cdot}0 \times 10^7\ \text{rad}^2\,\text{s}^{-1}$ (3500 rpm for 10 min), $R_{min} = 6{\cdot}2$, $R_{max} = 14{\cdot}4$ cm; 4 °C. The mitochondrial fraction (ML) was isolated by centrifugation of the cytoplasmic extract (E) in a HB-4 rotor at $\int_0^t \text{rpm}^2\,dt = 13 \times 10^8\ \text{rad}^2\,\text{s}^{-1}$ (10 000 rpm for 20 min), and the microsomal fraction (P) by centrifugation in a Beckman ultracentrifuge L5-75 (equipped with an integrator) with a Ti 50 rotor at $\int_0^t \text{rpm}^2\,dt = 5{\cdot}9 \times 10^{10}\ \text{rad}^2\ \text{s}^{-1}$ (40 000 rpm for 60 min), $R_{min} = 5{\cdot}7$, $R_{max} = 10{\cdot}8$ cm. The fractions were washed as shown in Fig. 1, and the washings were added to the corresponding supernatant. The final supernatant was termed the S-fraction and contains predominantly the cell's soluble material, i.e. cytosol.

Enzyme assays

Palmitoyl-CoA hydrolase activity (EC 3.1.2.2) was assayed by the release of [1-^{14}C]-labelled fatty acid from the radioactive CoA ester (Baardseth & Slinde, 1983*a*; Berge & Farstad, 1979).

Peroxidase activity (EC 1.11.1.7) was determined at 420 nm using guaiacol and hydrogen peroxide as substrate, as described by Lu & Whitaker (1974). Malate dehydrogenase (EC 1.1.1.37) and glutamate oxaloacetate transaminase (EC 2.6.1.1) activity were both determined at 340 nm by measuring the change in absorbance of NADH using analysis kits from Boehringer Mannheim, GmbH.

Catalase (EC 1.11.1.6) activity was measured at 230 nm using hydrogen peroxide, having an absorbance at 230 nm = 1·0, as substrate (Bergmeyer *et al.*, 1974). Cytochrome *c* oxidase activity (EC 1.9.3.1) was measured spectrophotometrically by following the oxidation of ferrocytochrome *c* at 550 nm (Yonetani & Ray, 1965). Cytochrome *c* was reduced by adding a few grains of solid dithionite. The reducing agent was removed by molecular sieve chromatography on a Sephadex G-10 column. Both catalase and cytochrome *c* oxidase activity were determined by measuring the initial reaction rate ($\Delta A\ \text{min}^{-1}$). A fixed concentration of H_2O_2 and cytochrome *c* were used. The values are therefore comparable.

All enzyme assays were carried out at 25 °C using a Shimadzu UV-300 spectrophotometer with a 1 cm cell.

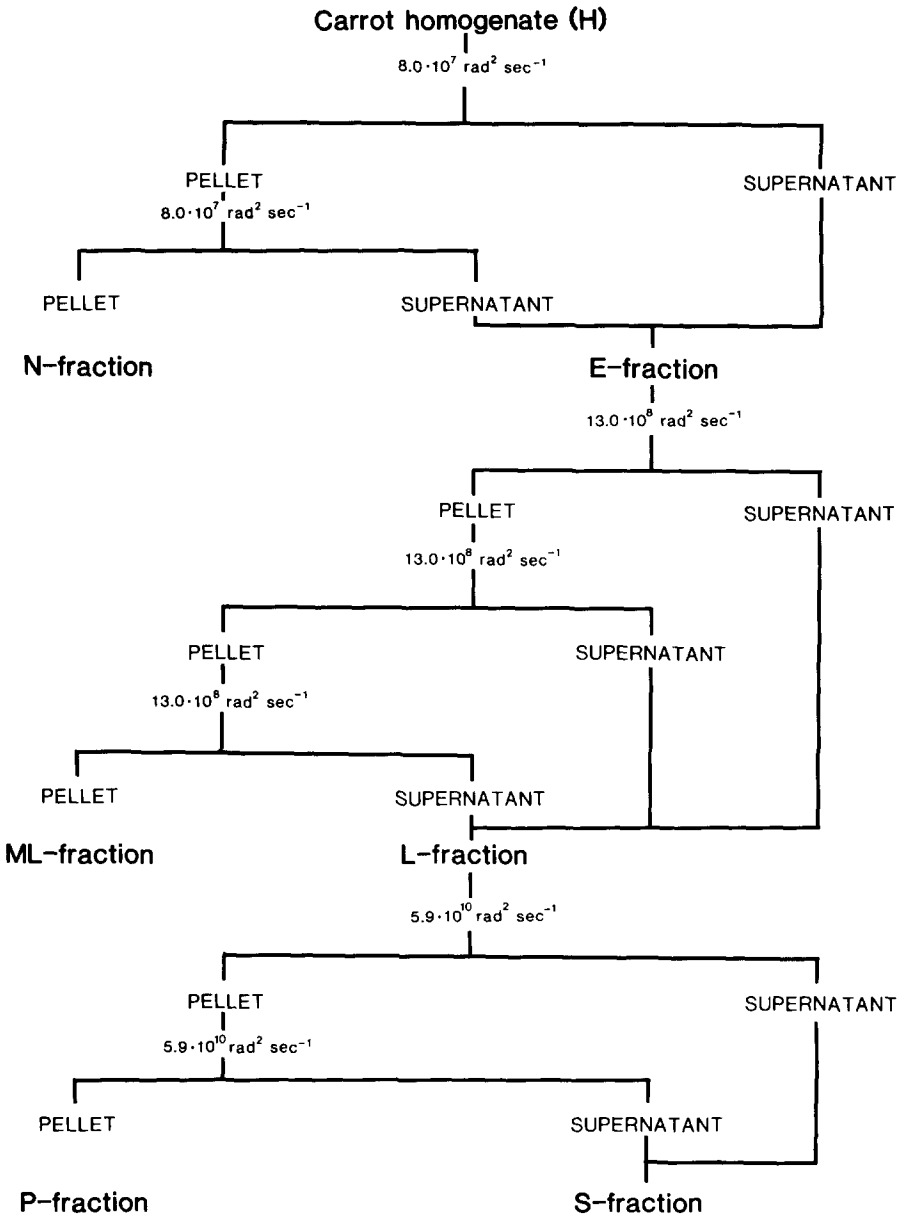

Fig. 1. Fractions and sedimentation integrals used in the differential centrifugation of carrot homogenate. The N-fraction and ML-fraction were obtained by using a Sorvall HB-4 rotor and the P-fraction using a Beckman Ti 50 rotor.

Determination of protein

Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard, or by measuring the absorbance at 280 nm (Thorne, 1978); $A_{280}^{1\,cm} = 1$ corresponds to 1 mg of protein per millilitre.

RESULTS AND DISCUSSION

Homogenization of carrot is difficult due to the toughness of the tissue (Bonner, 1967). The method of tissue breakage should be as gentle and rapid as possible. Experience has shown that mechanical methods based on rapid cutting, chopping or slicing of the tissue are the most likely to lead to the successful isolation of active, undamaged subcellular fractions from plant tissue (Leech, 1977). The Ultra-Turrax is a severe homogenization device, as opposed to the Robot Vertical Cutter, but the difference between these two methods was not reflected in the results presented in Table 1.

TABLE 1

Distribution of Palmitoyl-CoA Hydrolase and Some Marker Enzymes in Fractions Isolated by Differential Centrifugation of Carrot Homogenate (see (Fig. 1)

	Total activity	*n*	*Distribution* (%)				*Recovery* (%)
			N	*ML*	*P*	*S*	
Palmitoyl-CoA hydrolase	$(8 \pm 3) \times 10^4$	6	0	8±8	20±13	55±17	83±26
Malate dehydrogenase	7·7±1·6	6	0	1±2	2±3	84±24	87±27
Catalase	1·4±0·1	4	0	2±1	6±6	86±15	94±18
Peroxidase	0·8±0·2	5	0	1±2	3±4	58±14	62±12
Cytochrome *c* oxidase	10·1	1	15	104	0	0	119
Protein (A_{280})	9·7±1·2	6	4±4	9±5	35±7	32±8	80±5

The enzyme activities and the protein contents of the fractions are expressed as a percentage of the total in whole homogenate (E + N) ± standard deviations. N = nuclear fraction; ML = mitochondrial fraction; P = microsomal fraction; S = particle-free supernatant. *n* = number of experiments. Palmitoyl-CoA hydrolase activity is expressed as cpm, malate dehydrogenase, catalase, peroxidase and glutamate oxaloacetate, transaminase as $\Delta A\ min^{-1}$, cytochrome *c* oxidase as μmol per litre per minute and protein in milligrams per millilitre.

The centrifugal effects used in the present study were selected based on the osmolarity of the medium and the sedimentation coefficient used to obtain swede mitochondria (Slinde *et al.*, 1983). The aim was to generate a typical nuclear fraction, and a ML fraction containing, among other cell organelles, the mitochondria and peroxisomes.

The total activities and distribution of palmitoyl-CoA hydrolase, compared with the marker enzymes used, are shown in Table 1.

Protein was determined by measuring the absorbance at 280 nm, since the Folin-Ciocalteu's reagents gave recoveries which were too high (up to 500 % recovery). This is probably due to the presence of interfering compounds in the tissue (Peterson, 1979).

Cytochrome *c* oxidase, which is localized in the inner mitochondrial membrane (Tolbert, 1974), was found mainly in the ML fraction. The true activity of cytochrome *c* oxidase is difficult to establish, since the presence of ascorbate in carrots reduces the substrate cytochrome *c* (Minnaert, 1961). Malate dehydrogenase, which is localized in both the cytosol and the matrix of the mitochondria in potato (Ruis, 1971), was found mainly in the S-fraction.

Catalase, a marker enzyme for the peroxisomal and mitochondrial fraction in spinach leaf (Gerhardt, 1981), for microbodies in pea (McNeil & Thomas, 1976) and for peroxisomes in potato (van der Plas *et al.*, 1976), was found in the mitochondrial, the microsomal, but mainly in the cytosolic, fractions (Table 1). Day *et al.* (1979) found considerable catalase activity in the mitochondrial preparation from potato, but 95 % of this was lost upon washing the mitochondria, i.e. the catalase activity will mainly be found in the cytosolic fraction.

Peroxidase, which is located in the cell wall and Golgi bodies of potato (Brinkman & Smina, 1977), and in the cell wall of maize root tips (Parish, 1975) and peas (Ridge & Osborne, 1970), was detected mainly in the cytosolic fraction (Table 1), but with a low recovery (62 %).

Palmitoyl-CoA hydrolase was found in the ML-, P- and S-fractions. Its presence in the ML- and P-fractions indicates a membrane binding. In order to study the possible membrane-bound localization of the palmitoyl-CoA hydrolase activity, the ML fraction was subjected to freezing and thawing. The results in Fig. 2 show that glutamate oxaloacetate transaminase and malate dehydrogenase, both being matrix enzymes (Ruis, 1971; Macey, 1983), are released to the same extent. On the other hand, both palmitoyl-CoA hydrolase and peroxidase are found to stay in their respective membranes.

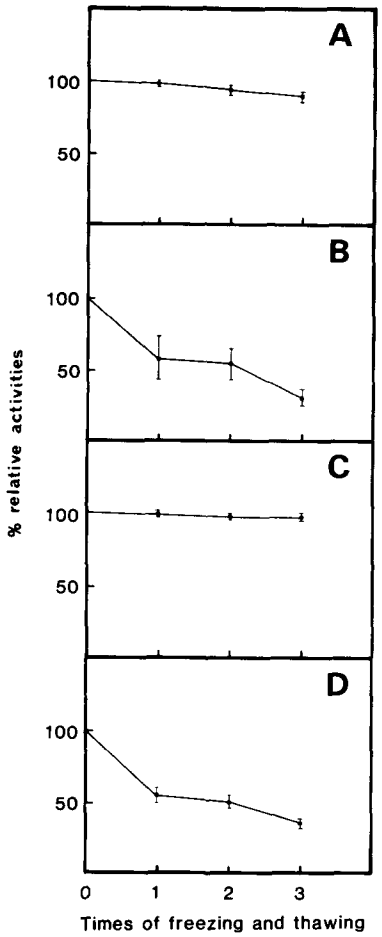

Fig. 2. The mitochondrial fraction (ML) was disrupted by freezing and thawing, and relative activities of palmitoyl-CoA hydrolase and marker enzymes were determined. A, Palmitoyl-CoA hydrolase; B, malate dehydrogenase; C, peroxidase; D, glutamate oxaloacetate transaminase.

The results show that homogenization disrupts the tissue, and a rather high amount of the palmitoyl-CoA hydrolase is found in the cytosol. However, both an endoplasmic (Table 1) and a heavier membrane localization (Fig. 2) is observed.

In comparison, the palmitoyl-CoA hydrolase activity in human platelets (Berge *et al.*, 1980) has the same distribution as found in carrots, while in human liver (Berge *et al.*, 1980) the activity was found in the microsomes and the mitochondrial matrix. The substrate specificity of the

assay has not been tested but earlier findings indicate that carrots contain more than one hydrolase (Baardseth & Slinde, 1983*a*).

When the palmitoyl-CoA hydrolase activity was measured in the xylem and phloem of the carrot, the ratio was 1·2:1, i.e. a somewhat higher activity will be released by cubing the carrot, compared with wounding the carrot, i.e. the phloem.

Since cell disruption and release of palmitoyl-CoA lead to the possibility of a higher free fatty acid level, it may be assumed that an increase in fatty acid oxidation takes places under these conditions. This again would increase the production of off-flavours; for example, aldehydes, through α-oxidation.

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