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Partial Purification and Characterization of the Mitochondrial and Peroxisomal Isozymes of Enoyl-Coenzyme A Hydratase from Germinating Pea Seedlings[1]

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ABSTRACT

Distinct organellar forms of the β-oxidation enzyme enoyl-coenzyme A (CoA) hydratase were partially purified and characterized from 2-day germinated pea (*Pisum sativum* L.) seedlings. The purification was accomplished by disruption of purified mitochondria or peroxisomes, $(NH_4)_2SO_4$ fractionation, and gel permeation chromatography using a column of Sephacryl S-300. The organellar isozymes had distinct kinetic constants for the substrates 2-butenoyl-CoA and 2-octenoyl-CoA, and could be easily distinguished by differences in thermostability and salt activation. The peroxisomal isozyme had a native M_r of 75,000 and appeared to be a typical bifunctional enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, while the mitochondrial isozyme had a native M_r of 57,000 and did not have associated dehydrogenase activity. Western blots of total pea mitochondrial proteins gave a positive signal when probed with anti-rat liver mitochondrial enoyl-CoA hydratase antibodies but there was no signal when blots of total peroxisomal proteins were probed.

The subcellular localization of β-oxidation of fatty acids in plant cells has been a point of some controversy. It was initially assumed that the β-oxidation sequence was localized within mitochondria (14), as was thought to be the case at that time in animal tissues. It was then demonstrated that in the endosperm of germinating castor oil seeds the β-oxidation enzymes were colocalized with the enzymes of the glyoxylate cycle within specialized peroxisomes (6). Subsequently, it was demonstrated that β-oxidation has a dual localization in animal cells: mitochondria and peroxisomes (18). However, despite occasional reports to the contrary (*e.g.* ref. 19), for many years the consensus has been that there is no β-oxidation in plant mitochondria and that this pathway in plant cells is localized exclusively within peroxisomes (reviewed in ref. 14).

Thomas and associates (21, 30, 35) reported that mitochondria from germinating pea seedlings were capable of the β-oxidation of fatty acids, and that this process was absolutely dependent upon added carnitine. Carnitine dependence is one characteristic of animal cell mitochondrial β-oxidation that distinguishes it from the peroxisomal pathway (18). It has been suggested that the pea mitochondrial β-oxidation activity observed by Wood and Thomas could be attributed to peroxisomal contamination (12, 20). This criticism ignores the fact that pea mitochondrial β-oxidation requires L-carnitine while the peroxisomal pathway is unable to utilize acylcarnitines (23, 34). Nevertheless, the criticism of peroxisomal contamination was directly addressed and, using an improved purification procedure, it was shown that pea mitochondria devoid of peroxisomal contamination (5) were still fully capable of carnitine-dependent fatty acid β-oxidation (31). Having established the validity of the mitochondrial localization of β-oxidation in peas, it was desirable to compare the physicochemical and catalytic properties of the organelle-specific isozymes. EH[3] was chosen for the initial studies because it has the highest *in vitro* catalytic activity among the β-oxidation enzymes.

MATERIALS AND METHODS

Plant Material

Pea seeds (*Pisum sativum* L. cv Bunting) were a gift from Batchelors Foods Ltd., Worksop, Notts, England. The pea seeds were imbibed in running tap water for 8 h and then germinated for 40 h in the dark at 25°C on moist blotting paper (21).

Reagents

Enzyme grade ammonium sulfate, molecular biology grade 2-mercaptoethanol, coupling enzymes and standard proteins, and BTP buffer were from the Sigma Chemical Company, Poole, Dorset, England. Acetoacetyl-CoA, the lithium salt of CoA, and NADH were supplied by PL Biochemicals. Crotonic anhydride and *trans*-2-octenoic acid were from the Aldrich Chemical Company, Gillingham, Dorset, England. All other reagents were of analytical grade.

Preparation of Enoyl-CoAs

trans-2-Butenoyl-CoA was prepared from crotonic anhydride as described by Miernyk and Trelease (23). *trans*-2-

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[3] Abbreviations: EH, enoyl-CoA hydratase (EC 4.2.1.17); BTP, bis-tris-propane buffer; EH_m, the mitochondrial isozyme; EH_p, the peroxisomal isozyme;

Octenoyl-CoA was prepared by the mixed anhydride method as described by Miernyk and Trelease (24). Both enoyl-CoAs were purified and quantitated as previously described (24).

Organelle Isolation

Mitochondria and peroxisomes were isolated from the cotyledons of 2-d germinated pea seedlings by the method of McNeil and Thomas (21) and then further purified by the method of Burgess *et al.* (5). Briefly, pea seedlings were homogenized with a mortar and pestle and organelle-enriched fractions prepared by rate-zonal sedimentation. The fractions were further enriched in either mitochondria or peroxisomes by resuspension, and resedimentation. Finally, the washed organelles were purified by rate-zonal sedimentation on continuous: discontinuous sucrose gradients. The resuspended washed-organelle fractions were applied to gradients consisting of steps of 4 mL of 60% (all sucrose concentrations are w/v) sucrose, 5 mL 51% sucrose, and 6 mL 44% sucrose, all layered beneath a 10 mL continuous gradient formed from 6 mL of 41% sucrose and 4 mL 31% sucrose. The gradients were centrifuged at 40,000*g* for 2 h in a Beckman SW-28 rotor.

Enzyme Assays

Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities were assayed as described by Miernyk and Trelease (23) except that 90 mM BTP buffers were used. Catalase and fumarase activities were assayed as described by Aebi (1) and Hill and Bradshaw (16), respectively. The results from initial-rate kinetic studies were analyzed by iterative curve fitting using nonlinear regression (11).

Enzyme Purification

The first step in purification of the isozymes was the isolation of purified organelle fractions, in order to minimize isozyme cross-contamination. The organelle fractions were diluted fivefold with BTP buffer (pH 7.5), containing 2 mM mercaptoethanol, then disrupted by two 15 s bursts with a Polytron homogenizer at a setting of 6. After removal of membranes by centrifugation, the isozymes were purified and concentrated by ammonium sulfate precipitation. The proteins in the ammonium sulfate pellets were further fractionated by gel permeation chromatography. The peak fractions from gel permeation were combined and concentrated by addition of ammonium sulfate to 80% of saturation. The peak enzyme fractions from gel permeation were stable during storage at 4°C for at least 1 week and, unless otherwise noted, were used in all catalytic analyses.

Measurement of Relative Molecular Mass

The native M_r for each isozyme was determined by gel permeation chromatography using a 95 × 2.5 cm column of Sephacryl S-300. The column equilibration buffer was 50 mM K-PO_4 (pH 6.9), containing 100 mM KCl and 2 mM 2-mercaptoethanol. The column was developed by upward flow at a rate of 80 mL/h. Blue dextrin was used to determine the void volume of the column and KCl was used to determine the included volume. Calibration was accomplished using the following standard proteins (mol wt): bovine liver catalase (240,000), porcine heart lactate dehydrogenase (140,000), bovine serum albumin (67,000), and bovine heart Cyt *c* (12,400).

Thermostability

Samples of the isozymes purified through the $(NH_4)_2SO_4$ precipitation step were adjusted to the same total and specific activity by addition of BSA from a stock solution in BTP buffer (pH 7.5), containing 2 mM mercaptoethanol. Aliquots of the isozymes in 1.5 mL microfuge tubes were held in a foam rubber float within a Grant Instruments (Cambridge) Ltd thermostatted circulating water bath set at 60°C. At specified intervals tubes were removed from the water bath and quickly transferred to an ice bucket. Residual enzyme activity was measured after at least 5 min on ice.

Salt Activation

The partially purified isozymes were desalted using a small column of Sepahdex G-10, poured in a 10 mL syringe barrel, and equilibrated with 10 mM BTP (pH 6.9), plus 2 mM 2-mercaptoethanol. After desalting, aliquots of the enzyme-containing fractions were preincubated for 2 min in assay buffer containing KCl at the indicated final concentrations. The other assay components were then added, and the reactions initiated with butenoyl-CoA.

Immunochemical Analyses

Protein purification and the preparation of antisera against rat liver mitochondrial enoyl-CoA hydratase (10), cotton seed catalase (17), and the *Brassica* mitochondrial pyruvate dehydrogenase complex (28) have been previously reported. Sample preparation, SDS-PAGE using 12.5% acrylamide gels, and immunodetection were described previously (22).

Other Analytical Methods

Protein concentrations were estimated by the method of Bradford (4), using purified fraction V BSA (21) as the standard.

RESULTS AND DISCUSSION

Purified organelle preparations from germinating pea seedlings were subjected to physical disruption, then assayed for marker (7) and β-oxidation enzyme activities. The activities of the β-oxidation enzymes were approximately equally distributed in both organellar fractions (Table I). This result is in marked contrast to reports from other workers on the localization of the β-oxidation enzymes in higher plant cells (6, 12, 15, 27), but is consistent with previous publications from this laboratory (21, 30, 32, 34, 35). It has been noted that the substrates of the β-oxidation enzymes do not easily cross the intact mitochondrial inner membrane, thus this membrane must be disrupted before detecting enzyme activity (34). When mitochondria isolated by centrifugation in sucrose density gradients are added to cuvettes for spectrophotometric enzyme assays, sufficient sucrose is carried along to maintain

Table I. *A Typical Distribution of Activities of Marker Enzymes and the β-Oxidation Enzymes in Subcellular Fractions Prepared from 2-d Germinated Pea Seedlings*

Enzyme	Activity	
	Mitochondria	Peroxisomes
	% of total[a]	
Fumarase	93	7
Catalase	<1	99
Enoyl-CoA hydratase	54	46
3-Hydroxyacyl-CoA dehydrogenase	50	50
Acetoacetyl-CoA thiolase	56	34

[a] Total activities (μmol/min) in the original homogenate were: fumarase, 235; catalase, 33218; enoyl hydratase, 3340; 3-hydroxyacyl-CoA dehydrogenase, 1318; thiolase, 519. Total recoveries, relative to the samples loaded onto the gradients, were: fumarase, 81%; catalase, 69%; enoyl hydratase, 96%; 3-hydroxyacyl-CoA dehydrogenase, 88%; thiolase, 103%.

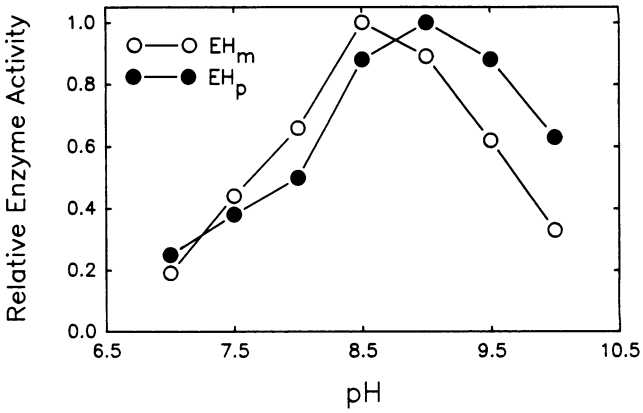

Figure 1. Activities of the pea seedling enoyl-CoA hydratase isozymes as a function of assay pH. The buffer used throughout was 90 mM BTP.

organelle integrity. The observation of negligible activity generally results in the addition of a larger sample that also supplies a greater concentration of osmoticum. It is then obvious that to adequately measure enzymes with membrane impermeant substrates, the membranes must be disrupted. Our results suggest that a reexamination of the earlier reports of subcellular localization of β-oxidation in higher plants is warranted. In addition to the necessity of membrane disruption, it should be noted that there is some evidence for tissue specific or developmental control of enzyme activity (*e.g.* thus far we have been unable to obtain any evidence for β-oxidation activity in mitochondria isolated from 14-d pea leaves).

Having established the occurrence of both mitochondrial and peroxisomal isozymes in 2 to 3 d germinated pea seedlings, a partial purification of the EH isozymes was accomplished by disruption of the organelle-enriched fractions, removal of membranous material by centrifugation, $(NH_4)_2SO_4$ fractionation, and gel permeation chromatography (Table II). The behavior of the two isozymes during salt precipitation was distinct (Table II). EH_m precipitated at a much narrower concentration range of $(NH_4)_2SO_4$ than EH_p, affording a significant purification at this step. Precipitation of EH_p resulted in a relatively small degree of purification, and this step was employed primarily to concentrate the protein prior to gel permeation chromatography. Both isozymes were purified approximately 10-fold by the gel permeation step (Table II). Overall, the mitochondrial isozyme was purified approximately 30-fold to a specific activity of 9.5 μmol/min/mg protein at a yield of 20%. The peroxisomal isozyme was purified approximately 10-fold to a specific activity of 23.2 μmol/min/mg protein but at a yield of only 4%. For acceptable recoveries during purification, it was important that a sulfhydryl-reducing agent such as 2-mercaptoethanol be included in all buffers from the organelle disruption step onwards. Initial efforts at $(NH_4)_2SO_4$-fractionation were hindered by what appeared to be an inhibitor in some batches of the salt.

A typical analysis of the pH-activity relationships of the pea EH isozymes is presented in Figure 1. Optimum *in vitro* activity of EH_p was at a slightly more alkaline pH than EH_m (pH 9.0). The pH curve for EH_p was always more broad than that of the mitochondrial enzyme. While the pH-activity relationship for *Escherichia coli* EH is quite broad (2), all

Table II. *Partial Purification of the Mitochondrial and Peroxisomal Isozymes of Enoyl-CoA Hydratase from Pea Seedlings*

S_{27}, a homogenate of pea seedlings clarified by centrifugation at 27,000*g*; AS, $(NH_4)_2SO_4$ fractions; S-300, combined peak fractions from gel permeation chromatography.

Fraction	Units	Total Protein	Specific Activity	Purification	Yield
	μmol/min	*mg*	*units/mg*	*-fold*	%
S_{27}	113	1.53	0.074		
Mitochondria	50.1	175.0	0.29	1	100
40–65% AS	25.1	25.8	0.97	3.3	50
S-300 peak	10.4	1.1	9.45	32.6	21
Peroxisomes	23.2	21.1	1.10	1	100
30–80% AS	10.3	4.9	2.09	1.9	32
S-300 peak	0.9	0.04	23.20	11.1	4

Table III. *Initial Rate Analyses of the Peroxisomal and Mitochondrial Isozymes of Enoyl-CoA Hydratase*

Data were analyzed by iterative curve-fitting using nonlinear regression. Values for K_m are μM and for V_m are μmol min^{-1}.

Substrate	EH_p		EH_m	
	K_m	V_{max}	K_m	V_{max}
trans-2-Butenoyl-CoA	61	49	75	45
trans-2-Octenoyl-CoA	85	26	119	12

eukaryotic EHs have alkaline pH optima. Although there are no published data on plant EH_m, the cotton EH_p has a pH optimum of 9.0 (24).

There have been only a few studies of the effect of substrate acyl-chain length upon the kinetic constants for plant EHs. With both the cotton (24) and cucumber (3) EH_ps, there was an increase in K_m and a decrease in V_{max} as the acyl-chain length of the 2-enoyl-CoAs increased. While only butenoyl-CoA and octenoyl-CoA were tested as substrates for the pea EH_p, a similar pattern of changes in the kinetic constants was observed (Table III). In mammalian mitochondria and bacterial systems, there are multiple forms of EH, each having a particular substrate specificity (2, 8, 33). The changes in kinetic constants seen with an increase in substrate chain length for the pea EH_m follow a pattern similar to that of EH_p (Table III), although there are significant differences in the absolute values.

The pea cotyledon EH isozymes could be easily distinguished by differences in the rate of activity loss when incubated at 60°C (Fig. 2). EH_m was essentially unaffected by incubation at 60°C for up to 1 min. In contrast, EH_p was rapidly inactivated, with a $t_{0.5}$ of approximately 35 s. Longer incubations or higher temperatures did result in inactivation of the mitochondrial isozyme (data not presented). It has been previously reported that mammalian EH_m is stable at 60°C (10, 29), while EH_p is completely inactivated in less than 2 min at this temperature (10). Frevert and Kindl (9) used a "partial heat denaturation" step during the purification of the

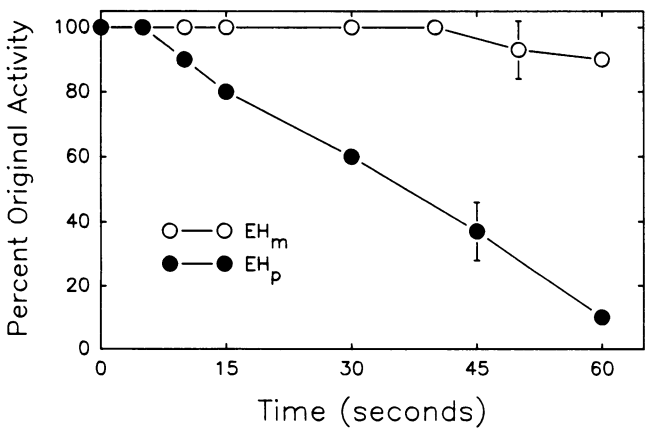

Figure 2. Stability of the partially purified pea seedling enoyl-CoA hydratase isozymes during incubation at 60°C. Data points are means ±SEM for three separate determinations.

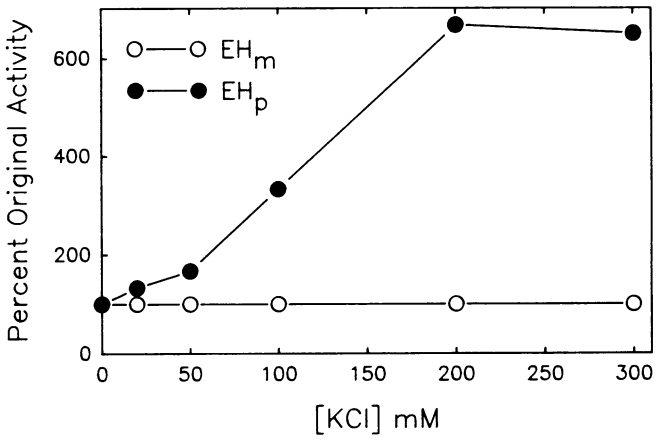

Figure 3. The effect of salt concentration upon the *in vitro* activities of the pea seedling enoyl-CoA hydratase isozymes.

cucumber cotyledon multifunctional protein, but no temperatures or times are presented.

Activity of the pea EH_p increased with increasing KCl concentration up to at least 200 mM, while there was no effect of KCl on the activity of the EH_m (Fig. 3). Activity of EH_p at 200 mM KCl was more than sixfold the activity at 0 KCl. There was a similar activation of EH_p when NaCl or $(NH_4)_2SO_4$ was used in place of KCl (data not presented). Our results are similar to those obtained by Furuta *et al.* (10) when studying the mitochondrial and peroxisomal EH isozymes from rat liver.

When the peroxisomal β-oxidation enzymes were initially characterized, it was observed that EH and 3-hydroxyacyl-CoA dehydrogenase activities were both associated with a single monomeric protein having a M_r of 71,000 to 75,000 (9, 26). It was subsequently reported that this multifunctional β-oxidation protein also has 3-hydroxyacyl-CoA epimerase activity (3). Using the column calibration standards shown in Figure 4, it was calculated that the M_r of pea seedling EH_p is 75,000, a value identical with that reported for other plant EH_ps (9). When the same fractions were assayed for 3-hydroxyacyl-CoA dehydrogenase activity, it was found that the two activities were exactly coincident (data not presented). In contrast, the M_r value calculated for the pea EH_m is 57,000 (Fig. 4). This is substantially smaller than the M_r value reported for mammalian mitochondrial EH (29) but is similar to the recently described enzyme from *Caulobacter crescentus* (25). When the fractions from the mitochondrial preparation were assayed for 3-hydroxyacyl-CoA dehydrogenase activity, it was observed that, while there was some overlap with EH, the two activities were not coincident (data not presented). Gross (13) recently reported that gel permeation chromatography could be used to separate EH and 3-hydroxyacyl-CoA dehydrogenase activities in preparations from mitochondria of the alga *Cyanidium caldarium*. The native M_r for the algal EH was 87,000, considerably smaller than the mammalian mitochondrial enzyme but larger than that from pea mitochondria.

Comparisons between the rat liver EH_m and EH_p have been made at both the protein and cDNA levels. While these

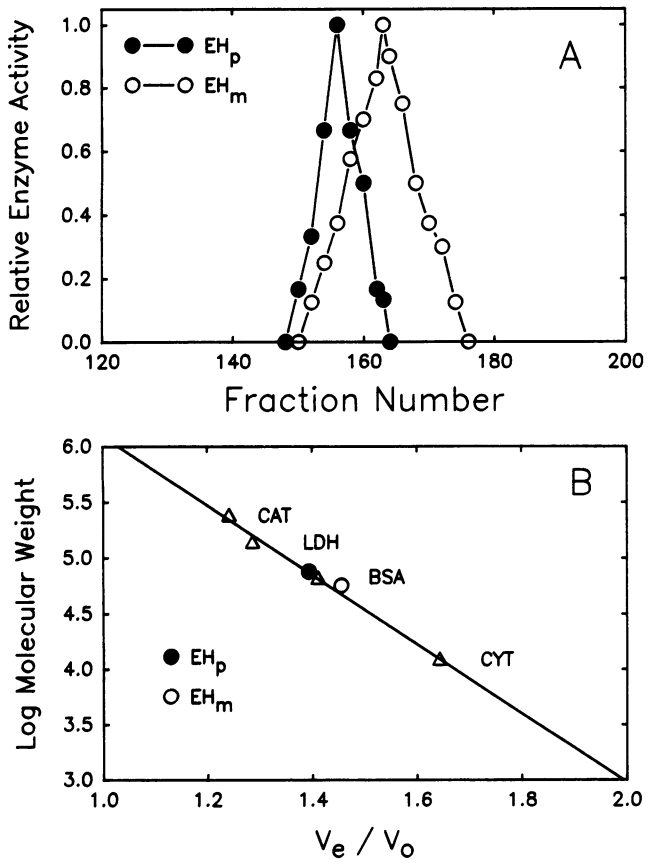

Figure 4. Determination of the relative molecular mass of the pea seedling enoyl-CoA hydratase isozymes. The elution profiles of the partially purified isozymes from the Sephacryl S-300 column are presented in panel A. The relationship between the elution volumes of the pea isozymes and those of calibration standards is presented in panel B.

isozymes have some similarities in physicochemical properties and some regions of primary sequence homology, they are clearly distinct gene products. Antibodies to the mammalian mitochondrial enzyme do not recognize the peroxisomal multifunctional protein. As neither pea EH has yet been purified to homogeneity, we attempted to make some structural comparisons using the rat liver antibodies. Both mitochondrial and peroxisomal fractions showed complex patterns when analyzed by SDS-PAGE with Coomassie blue staining (not shown). Purity of the fractions was evaluated using antibodies to the *Brassica* mitochondrial pyruvate dehydrogenase complex and cotton seed catalase, a typical peroxisomal marker enzyme. Anticatalase antibodies gave a signal only with the peroxisomal fraction (Fig. 5, d and e), while the anti-pyruvate dehydrogenase antibodies gave a strong signal with the mitochondrial fraction and a much reduced signal with the peroxisomal fraction (Fig. 5, b and c). We conclude from the marker enzyme activity plus immunochemical results that while the peroxisomal fraction is slightly contaminated with mitochondria, the mitochondria are not significantly contaminated with peroxisomes. When both fractions were probed with anti-rat liver EH_m antibodies, there was a signal only from the pea mitochondria. The reaction with the heterologous antibodies provides additional evidence for a distinct plant mitochondrial isozyme. From the Western analysis it can be seen that the putative pea EH_m has approximately the same SDS-PAGE mobility as cotton catalase (*cf.* Fig. 5, e and f). It has been reported that cotton seed catalase has a subunit M_r of 57,000 (17), and this same value was estimated for the native M_r of pea EH_m (Fig. 4B). These data suggest that the pea mitochondrial enzyme is active as a monomer.

CONCLUSIONS

The differences in physicochemical and catalytic properties of pea EH are consistent with the proposal that the organellar isozymes are distinct proteins. Although preliminary, the results of this enzymological study fully support the results of previous cell fractionation studies. There is considerable evidence for the occurrence of both mitochondrial and peroxisomal β-oxidation in animal, yeast, and some algal (13, 36) cells. It is increasingly obvious that, in at least some instances, higher plant cells must be included in this list. While there is clearly mitochondrial β-oxidation in cotyledons of germinating pea seedlings, we have been unable to detect any mitochondrial activity in mature, fully expanded pea leaves or in roots. This suggests that the expression of the mitochondrial

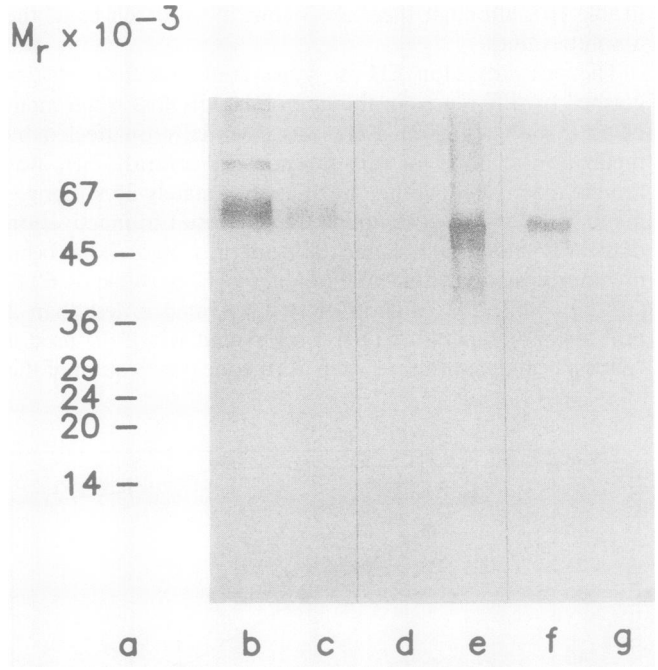

Figure 5. Western blot analysis of purified pea seedling organellar fractions. Lane a indicates the M_r for the standard proteins: BSA, 67,000; ovalbumin 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,200. Lanes b, d, and f are total mitochondrial proteins while lanes c, e, and g are total peroxisomal proteins. Lanes b and c were probed with anti-pyruvate dehydrogenase antibodies, lanes d and e were probed with anti-catalase antibodies, and lanes f and g were probed with anti-rat liver EH_m antibodies.

β-oxidation is subjected to some sort of tissue-specific or developmental control.

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