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EFFECT OF AGEING ON ENZYMES OF PHENYLPROPANOID METABOLISM IN *SOLANUM TUBEROSUM* DISCS

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Abstract—Separation of cell fractions or cell organelles of potato tuber by differential centrifugation and by sucrose density gradient centrifugation showed that, in dormant tissue, nearly all the activity of shikimate and prephenate dehydrogenases, phenylalanine ammonia lyase, cinnamate-4-hydroxylase and an *O*-methyltransferase for caffeate was in the soluble fraction. All these enzymes increased in activity in slices aged in light for 18 hr. In contrast to the other enzymes, cinnamate hydroxylase becomes associated with the microsomal fraction in aged discs.

INTRODUCTION

IN WASHED potato slices maintained in light there is a marked increase in the levels of chlorogenic acid[1] and an enzyme associated with its biosynthesis, namely, phenylalanine ammonia lyase[2,3] (PAL, E.C. 4.3.1.5). Other enzymes of phenylpropanoid metabolism, such as cinnamate-4-hydroxylase and *O*-dihydric phenol methyltransferase have been shown to increase significantly in response to illumination in parsley cell cultures.[4] Cinnamate-4-hydroxylase has been shown to be microsomal in a number of tissues[5–7] whereas PAL is present in peroxisomes of spinach,[8] glyoxysomes of *Ricinus* endosperm[9] and absent from the peroxisome-like structures in tuber cells.[10] In a continuing study of phenylpropanoid metabolism in storage tissue[3] we report here data on the intracellular distribution of some of the relevant enzymes in both dormant and aged slices of potato tuber.

RESULTS

Characterization of the Various Subcellular Fractions

The main subcellular fractions examined were obtained by centrifuging a potato brei at 10 000 *g* (crude organelle pellet) and at 100 000 *g* (microsomal pellet). The 100 000 *g* supernatant was assumed to contain only soluble proteins. Electron microscopy of the 10 000 *g*

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pellet revealed mitochondria and microbodies (peroxisomes). No such particles were present in the other fractions.

After sucrose density fractionation of the 10 000 *g* fraction, 4 major peaks of protein were identified on the gradient profiles. These included a peak at high specific gravity corresponding in density and in the presence of catalase to microbodies; another peak corresponding in density and the presence of fumarase to mitochondria[11] and an intermediate peak which increased in size in a preparation in which browning had occurred, and which possibly is an aggregation of microbodies and mitochondria. The identity of a fourth peak, at low specific gravity, was not investigated.

TABLE 1. INCREASE IN ACTIVITY OF SHIKIMIC ACID: NADP OXIDOREDUCTASE (SOR) AND PREPHENIC ACID: NADP OXIDOREDUCTASE (POR) ON AGEING IN LIGHT OF 'NETTED GEM' POTATO TUBER TISSUE

Enzyme	Activity (μmol/hr/g fr.wt) Dormant	Aged	% Increase
SOR	0·524	1·17	220
POR	0·250	0·626	250

Localization and Levels of Enzymes Concerned with Phenolic Metabolism

Shikimic dehydrogenase (shikimic acid : NADP oxidoreductase (SOR)) was completely soluble and not associated with any particulate fraction. The activity increased more than 2-fold during the incubation period (Table 1). Prephenic dehydrogenase (prephenic acid: NADP oxidoreductase (POR)) was also soluble and found to increase on incubation (Table 1).

TABLE 2. SUBCELLULAR DISTRIBUTION OF PHENYLALANINE AMMONIA LYASE FROM POTATO TUBER TISSUE AGED IN THE LIGHT

	var. 'Netted Gem' Expt. 1		Expt. 2		var. 'Norgold'	
Cell fraction	Activity*	%	Activity*	%	Activity†	%
Organelle	13·9	2	7	4	260	1·6
Microsome	8·8	5	3	5	240	1·5
Soluble	16·4	93	156	94	15 400	96·9

* μmol cinnamic acid formed/hr/g fr. wt.
† dpm in cinnamic acid/hr/g fr. wt.

The activity of PAL was too low in dormant potato to be accurately measured spectrophotometrically. While about 90% of the activity recovered from aged potato was found in the soluble phase (Table 2), the particulate fractions did contain some activity. When the organelle-containing fraction (the 10 000 *g* pellet) was examined on a sucrose-density gradient, PAL was found to be associated with all the protein-containing peaks except the

[11] HUANG, A. and BEEVERS, H. (1971) *Plant Physiol.* **48**, 637.

one corresponding to the peroxisomes. It was not removed by washing of the organelle or microsomal pellets (Table 3).

TABLE 3. PHENYLALANINE AMMONIA LYASE ACTIVITY IN UNWASHED AND WASHED SUBCELLULAR FRACTIONS FROM 'NORGOLD' POTATO TUBER TISSUE AGED IN THE LIGHT

Cell fraction	Original activity (dpm/hr)	After washing (dpm/hr)	
Organelle	18 000 ± 950	Particulate	10 300 ± 2000
		Soluble	6500
Microsomal	16 800 ± 4120	Particultae	5520
		Soluble	4210 ± 1300
Soluble	(1·075 ± 0·06) × 10^6		

While cinnamic acid hydroxylase was found in all fractions in dormant potato, most of the activity was recovered in the soluble fraction (Table 4). In aged potato, however, most of the activity was recovered in the microsomal fraction. Enzyme activity increased about 220% on ageing in light.

TABLE 4. SUBCELLULAR DISTRIBUTION OF CINNAMIC ACID 4-HYDROXYLASE FROM DORMANT AND LIGHT-AGED TUBER TISSUE FROM 'NETTED GEM' POTATOES

Cell fraction	Dormant tissue Activity*	%	Aged tissue Activity*	%
Organelle	0·165	2·4 ± 0·2	3·16	20·4 ± 3·1
Microsomal	0·900	13·7 ± 4·6	9·76	67·3 ± 4·0
Soluble	5·52	84·0 ± 20·0	1·17	12·2 ± 0·72
	6·59		14·7	

* Activity in μmol/hr/g fr. wt.

p-Coumaric acid hydroxylase could not be detected in our potato tuber tissue, despite the fact that four methods were used. A soluble enzyme which catalyzed the *O*-methylation of caffeic acid was found to increase 240% as the tissue aged in light (Table 5).

Administration of Tyrosine-^{14}C and Phenylalanine-^{14}C to Subcellular Fractions of Aged Potato Tuber

On feeding phenylalanine-2-^{14}C to the organelle fraction, it was only in the microsomal fraction and the soluble fraction that radioactive cinnamic acid was recovered. Radioactive *p*-coumaric acid was not detected but this is to be expected as cinnamic acid hydroxylase requires the presence of NADPH.

In aged potato there was no trace of tyrosine ammonia-lyase activity. Radioactive *p*-hydroxyphenylacetic acid (PHPA) and *p*-hydroxyphenylpyruvic acid (PHPP) were formed

from administered tyrosine-2-^{14}C. The microsomal fraction formed very little PHPA but it did form PHPP, while the organelle fraction and the soluble fraction were shown to be capable of forming both compounds.

TABLE 5. SUBCELLULAR DISTRIBUTION OF AN *O*-METHYLTRANSFERASE ACTING ON CAFFEIC ACID IN DORMANT AND LIGHT AGED POTATO TUBER TISSUE FROM 'NETTED GEM' POTATOES

Cell fraction	Dormant tissue Activity*	Dormant tissue %	Aged tissue Activity*	Aged tissue %
Organelle	0·009	4·7 ± 0·98	0·015	2·9 ± 0·84
Microsomal	0·004	2·1 ± 0·47	0·008	1·4 ± 0·21
Soluble	0·185	93·2 ± 16·0	0·453	95·6 ± 15·3
	0·198		0·476	

* μmol/hr/g fr. wt.

DISCUSSION

Increases in activity in tissue aged in the light were noted in the five enzymes of phenolic metabolism that were studied. PAL showed the greatest increase which suggests that it might be limiting in phenolic biosynthesis in dormant potatoes. Chlorogenic acid, a quinic acid ester of caffeic acid, is one of the main compounds accumulating in potato upon ageing,[1] and our inability to detect an enzyme hydroxylating *p*-coumaric acid to caffeic acid is surprising. An enzyme for hydroxylating *p*-coumarate is present in the peel of 'Kennebec' potatoes;[12] perhaps it is absent from the peel-free tuber tissue which was used here. Alternatively, hydroxylation might occur only at the level of the quinic acid ester of *p*-coumaric acid.

PAL occurs both in the particulate and soluble fractions. Although Schopfer[13] has suggested that the presence of PAL in particles may be attributed to artifacts that form when PAL is treated with dilute buffer, the enzyme remained in microsomal particles of buckwheat after washing[7] and in the peroxisomes of spinach after resedimentation.[8] Similarly, the present study shows that even washed particulate fractions from potato contain PAL activity (Table 3). The evidence for a particulate form of PAL seems convincing.

Despite this evidence for localization of PAL in various organelles, 90% of the enzyme recovered from *Ricinus* endosperm is in the soluble phase,[9] and a similar distribution exists in potato. Unless the enzyme associated with the particles is vastly more active than the soluble enzyme, the bulk of the catalytic activity must be carried out in the cytosol. Similarly, the activity, if any, of *O*-methyltransferase, and cinnamic acid hydroxylase in dormant potatoes probably takes place in the soluble phase. The cinnamic acid hydroxylase from aged potatoes is largely particulate and in this respect its distribution agrees with what has been found in other investigations of actively metabolizing tissues.[5–7]

The reactions involved in the metabolism of tyrosine seem to be compartmentalized also. The oxidative deamination of tyrosine appears to be mainly microsomal, although some conversion takes place in the organelle fraction. This fraction is also the site of the most active decarboxylation of PHPP to PHPA. In *Ricinus* both reactions take place in the pro-

[12] PATIL, S. S. and ZUCKER, M. (1965) *J. Biol. Chem.* **240**, 3938.
[13] SCHOPFER, P. (1971) *Planta* **101**, 339.

plastids and glyoxysomes.[14] Since the 10 000 *g* fraction of potato contains an organelle analogous to the glyoxysome, it well might be that it is in this organelle of the potato that decarboxylation of PHPP takes place.

If the intracellular localization of enzymes as determined by these techniques reflects the situation *in vivo*, then there must be a flux of substrate molecules out of the cytosol onto particles, off again and on to different sites of reaction. If this does represent the situation *in vivo*, then perhaps the different sites of the enzymes are subject to different controls. In the liver of the early fetus of the Rhesus monkey, tyrosine transaminase is mainly mitochondrial, while in the later fetus, a soluble form is also present.[15] These forms are subject to different control mechanisms, reflecting the different needs of the fetal tissue at the different times. In the potato it may be that the enzymes associated with different organellel or the two different forms of cinnamate hydroxylase are subject to different contros mechanisms.

Cinnamate hydroxylase is one of the three enzymes (PAL, and *p*-coumaric acid : CoA ligase are the other two) which undergo a parallel increase in activity in parsley cell culture after exposure to light.[4] Zucker[16] suggests that these enzymes may be formed by an operon, and that cinnamate hydroxylase originates as a soluble enzyme which then assumes a microsomal form. The present work suggests that in the case of cinnamate hydroxylase, the transition between soluble and particulate is a controlled reaction. While the three enzymes mentioned above may in fact form an operon, the final situation in the cell is the result of more than one controlling reaction.

EXPERIMENTAL

Preparation of potato tissue. Cylinders were cut from tubers of potato variety 'Netted Gem'. 2 mm discs were cut from these and rinsed 20 min under running water. 5 g tissue and 5 ml H_2O were dispensed per Petri dish and discs were incubated at 20° for 18 hr under fluorescent lights.

Preparation of cell fractions. Discs were chopped in an onion chopper for 15 min with an equal vol. of buffered sucrose medium.[10] The brei was filtered through Miracloth and centrifuged for 5 min at 270 *g*. The supernatant was centrifuged at 10 000 *g* for 20 min to yield the crude organelle pellet. The supernatant from this was centrifuged for 1 hr at 40 000 rpm in a 50 Ti rotor in a Spinco Model L2-65B preparative ultracentrifuge. The supernatant from this was termed the soluble fraction, and the resuspended pellet was termed the microsomal fraction. Washing was carried out on subcellular fractions of 'Norgold' potatoes isolated in the above manner. Particulate fractions were homogenized with a Potter homogenizer in 10–20× the vol. of fresh isolation medium. Centrifugation was carried out as before. For investigation of the organelle fraction, the 10 000 *g* pellet from the above procedure was resuspended in fresh medium and layered gently on a discontinuous sucrose gradient. A typical gradient consisted of 5 ml 70% sucrose (w/v), 10 ml 60% sucrose (w/v), 10 ml 51% sucrose (w/v) and 8 ml 44% sucrose (w/v) in a 25 × 87 mm cellulose nitrate tube. The solutions used for gradients contained sucrose, 0·05 M tricine buffer pH 7·5, and 1 mM EDTA. On top of this was layered 6 ml of resuspended pellet. The tubes were centrifuged for 18 hr in a SW27 rotor in a Spinco Model L2-65B preparative ultracentrifuge. Two ml fractions were collected from the bottom of the tube via a capillary attached to an LKB Variaperpex pump. Refractive indices were measured with a Bausch and Lomb Abbé refractometer.

Electron microscopy of cell fractions. Aliquots of the resuspended organelle fraction and the microsomal fraction, and of the soluble fraction were passed through Millipore filters, pore size 0·22 μ. Fixation and staining for catalase were after Parish.[17] All procedures were carried out on the filter, which dissolved in the dehydrating MeOH, leaving the organic fraction as an easily handled sheet. Embedding and sectioning were by standard methods.

Enzyme assay. Catalase determinations were after the method of Aebi.[18] Fumarase was determined by the

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method of Racker.[19] Shikimic acid: NADP oxidoreductase was determined by the method of Balinsky and Davies.[20] Prephenic acid: NADP oxidoreductase was determined by the method of Gamborg,[21] using prephenic acid generated from the barium salt, a gift of Dr. O. Gamborg (Prairie Regional Laboratories, National Research Council, Saskatoon). Phenylalanine ammonia-lyase was measured spectrophotometrically by the method Zucker[2] or by the formation of cinnamic acid-1-^{14}C from phenylalanine 1-^{14}C. Cinnamic acid 4-hydroxylase was measured by the method of Russel.[12] The activity of the *O*-methyltransferase acting on caffeic acid was measured by an adaptation of the *O*-methyltransferase assay of Basmadjian and Paul.[22] In this case, the reaction mixture was acidified before extraction of the ferulic acid into Et_2O. The identity of the reaction product was established as ferulic acid by co-chromatography with authentic ferulic acid in 3 solvents. *p*-Coumaric acid hydroxylase was assayed by the method of Sato,[23] Nambudiri *et al.*[24] (using media free of sulfhydryl reagents), Stafford and Dresler[25] and a modification of the cinnamic acid 4-hydroxylase assay of Russel,[12] using *p*-coumaric acid as the substrate. Protein was measured by the absorbance 280/260 method.[26]

Administration of radioactive phenylalanine and tyrosine to subcellular fractions. Aged potato discs were separated into an organelle fraction, a microsomal fraction and a soluble fraction. Tyrosine-2-^{14}C (50 μCi/μmol, 0·5 μCi/feeding) and phenylalanine-2-^{14}C (16·5 μCi/μmol, 0·5 μCi/feeding) were administered to resuspended aliquots of each fraction. After 1 hr of incubation, the fractions were acidified to pH 2, extracted with Et_2O, and the extracts chromatographed in two dimensions in cellulose thin layer plates, in C_6H_6–HOAc–H_2O (10 : 7 : 3, upper phase), and in 2% HCO_2H.

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