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CYTOPLASMIC MEMBRANE SENESCENCE IN BEAN COTYLEDONS

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Key Word Index—*Phaseolus vulgaris*; leguminosae; cotyledon; microsomal enzymes; senescence; germination.

Abstract—Distinguishable patterns of cytoplasmic membrane senescence in cotyledon tissue of *Phaseolus vulgaris* have been elucidated by examining the behavior of four microsomal enzymes—NADH–cytochrome C reductase, NADPH–cytochrome C reductase, glucose-6-phosphatase and 5′-nucleotidase during germination. For young cotyledon tissue, specific activities for the phosphatases were similar for rough and smooth microsomal fractions, but both cytochrome C reductases were 2–3 times more concentrated in the smooth fraction. These proportionalities changed with increasing age. As senescence becomes more intense the enzyme activities change independently of one another. These changes do not appear to be influenced by the presence or absence of ribosomes on the membranes. Parallel analyses of phospholipid levels in the isolated fractions revealed that loss of microsomal enzyme activity correlates with an ultimate dismantling of the membranes into their macromolecular constituents. The data have been interpreted as indicating that functionally distinct membranes or regions of the same membrane are differentially sensitive to senescence.

INTRODUCTION

Cotyledon tissue undergoes modifications during germination that render it peculiarly suited as a system for the study of senescence. Metabolic activity, for example, is generally high in the young cotyledons but then declines progressively [1–8]. Protein bodies and starch, the major forms of nutrient storage in the young tissue, are hydrolyzed and the products of hydrolysis are translocated to the developing embryo [9]. The organelles of the storage cells are initially structurally and functionally intact. However, as germination advances the cytoplasmic structure deteriorates [5] and even the appearance of the cotyledons changes [9]. All of these events end in complete autolysis of the cell cytoplasm and death of the tissue.

Previous studies with cotyledons of *Phaseolus vulgaris* have demonstrated that membranes of the storage cells acquire changes in their properties during germination that are symptomatic of senescence. Isolated plasma membranes from senescent tissue characteristically have lower cation-sensitive ATPase activity than do corresponding membranes from young cotyledons, as well as a reduced capacity for ATP-dependent cation transport [10]. Smooth microsomal membranes also show lower enzymatic activities as germination progresses [11]. In the present study, an effort has been made to characterize patterns of cytoplasmic membrane senescence in the tissue. Initially the manner in which senescence of the cotyledons influences the distributions of membranous enzymes between rough and smooth microsomal subfractions was determined. Subsequently, changes in the activities of these enzymes in microsomal subfractions were compared during germination to determine whether the senescence of distinguishable membrane types is synchronized.

RESULTS

Purity of the microsomal subfractions. The resolution of microsomes into their rough and smooth components involves separating vesicles of membrane derived from rough endoplasmic reticulum,

Table 1. RNA-P:Phospholipid-P ratios in preparations of rough and smooth microsomal membranes isolated from cotyledons of *Phaseolus vulgaris*

Tissue age (days)	Expt	μg RNA P/μg phospholipid P Rough microsomal subfraction	Smooth microsomal subfraction
2	A	1·31	0·14
	B	1·58	0·15
4	A	1·05	0·12
	B	1·33	0·17
7	A	2·93	0·08
	B	1·85	0·10
9	A	2·64	0·21
	B	0·50	0·16

Table 3. Changes during germination in total phospholipid of smooth and rough microsomal fractions

Tissue age (days)	μg Phospholipid P per 20 g cotyledon tissue Smooth microsomes	Rough microsomes
2	840 ± 43	371 ± 24
4	337 ± 40	187 ± 22
7	236 ± 34	117 ± 27
9	134 ± 31	137 ± 29

Standard errors are indicated; n = 3–5.

whose surfaces are studded with ribosomes, from those bearing no ribosomes and derived primarily, although not exclusively, from the smooth endoplasmic reticulum. The extent to which this separation was achieved in this investigation was monitored by determining RNA-P to phospholipid-P ratios in the rough and smooth subfractions (Table 1). Values for the ratio in smooth microsomes ranged from 0·08 to 0·21 indicating that the fraction typically contained less than 17% RNA-P and was predominantly smooth surfaced membrane. Corresponding values for rough microsomes were markedly higher. This supports the contention that this latter subfraction contained primarily rough surfaced membrane, although it is possible that some free ribosomes were also present.

Distributions of enzymes between rough and smooth microsomes. It has been previously reported that the enzymes NADH–cytochrome C reductase (reduced NAD:cytochrome C oxidoreductase, EC 1.6.99.3), NADPH–cytochrome C reductase (reduced NADP:cytochrome C oxidoreductase, EC 1.6.99.1), glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) and 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) are all associated with microsomal membranes from this tissue [7,11,12]. The cytochrome C reductases were assayed in the presence of cyanide and rotenone thus precluding their derivation from mitochondrial inner membrane [13]. NADH–cytochrome C reductase and NADPH–cytochrome C reductase were quantitatively preponderant in the smooth microsomal fraction as compared with the rough (Table 2). The data for 2-day old tissue are probably the most meaningful indication of distributions prior to the tissue being influenced by cytoplasmic autolysis. For both glucose-6-phosphatase and 5′-nucleotidase higher proportions of total microsomal activity were also found in the smooth microsomal subfraction. This was true for all ages of tissue examined, although during germination actual values for the proportions of glucose-6-phosphatase varied quite independently of those for 5′-nucleotidase (Table 2).

Table 2. Changes during germination in the distribution of enzyme activities between rough and smooth microsomal subfractions

Tissue age (days)	Microsomal subfraction	NADH–cytochrome C reductase	NADPH–cytochrome C reductase	Glucose-6-phosphatase	5′-Nucleotidase
2	Smooth	82·5 ± 3·0	88·2 ± 2·6	70·4 ± 6·5	65·2 ± 1·6
	Rough	17·5 ± 3·0	11·8 ± 2·6	29·6 ± 6·5	34·8 ± 1·6
4	Smooth	95·5 ± 0·8	75·3 ± 7·7	55·7 ± 4·4	63·0 ± 7·2
	Rough	4·5 ± 0·8	24·7 ± 7·7	44·3 ± 4·4	37·0 ± 7·2
7	Smooth	86·1 ± 1·7	55·7 ± 1·3	50·2 ± 2·4	70·4 ± 3·7
	Rough	13·9 ± 1·7	42·3 ± 1·3	49·8 ± 2·4	29·6 ± 3·7
9	Smooth	72·5 ± 8·1	61·1 ± 4·6	71·3 + 3·8	70·2 ± 3·6
	Rough	27·5 ± 8·1	38·9 ± 4·6	28·7 ± 3·8	29·8 ± 3·6

Activities are expressed as a percentage of total microsomal activity.
Standard errors are indicated; n = 3 or 4.

Table 4. Changes during germination in cytochrome C reductase specific activities of smooth and rough microsomal membranes

Tissue age (days)	NADH–cytochrome C reductase		NADPH–cytochrome C reductase	
	Smooth microsomes	Rough microsomes	Smooth microsomes	Rough microsomes
2	0·32 ± 0·06	0·15 ± 0·04	30·0 ± 5·0	9·7 ± 3·2
4	0·53 ± 0·06	0·05 ± 0·01	6·2 ± 0·7	3·2 ± 0·4
7	0·13 ± 0·04	0·03 ± 0·01	1·7 ± 0·2	2·1 ± 0·3
9	0·07 ± 0·02	0·02 ± 0·01	2·7 ± 0·6	1·6 ± 0·3

Activities are expressed as μmol reduced cytochrome C per μg phospholipid P per hr for NADH–cytochrome C reductase and as nmol reduced cytochrome C per μg phospholipid P per hr for NADPH–cytochrome C reductase. Standard errors are indicated; $n = 3$ or 4.

The higher proportions of total microsomal activity in the smooth subfraction could in part reflect disproportionate amounts of membrane in the two subfractions, but they could also result from differences in the abundance of enzyme per unit of membrane area. Measurements of total phospholipid-P in the subfractions indicated that, at least for 2-day-old tissue, the smooth fraction does contain more membrane than the rough. The level of phospholipid-P in the smooth subfraction was more than twice that for the rough at this stage of germination (Table 3). Moreover, the sp. act. of both cytochrome C reductases proved to be 2–3 × higher in the smooth fraction than in the rough for 2-day-old tissue, indicating that these enzymes are more abundant per unit area of membrane on the smooth surfaced membrane (Table 4). By contrast the sp. act. of the phosphatases in the two subfractions were of comparable magnitude for 2-day-old tissue (Table 5). Thus the asymmetric distributions of phosphatase activities at this stage in the germination sequence are simply a manifestation of disproportionate amounts of membrane in the two subfractions, whilst the more pronounced asymmetry evident for the cytochrome C reductases reflects the added factor of a higher enzyme activity per unit of membrane area for the smooth membranes.

Behavior of rough and smooth microsomal enzymes during germination. Measurements of cytochrome C reductases in smooth microsomes on the basis of tissue weight revealed that the activities of both enzymes progressively decline with advancing germination, particularly between the second and fourth day after planting (Table 6). On a percentage basis, NADPH–cytochrome C reductase falls off much more steeply between days 2 and 4 than does NADH–cytochrome C reductase, although both enzymes ultimately drop to very low levels such that by the 9th day of germination they are barely detectable. Somewhat similar patterns of change for these two enzymes were also evident for rough microsomes. However, in this case the activities of both enzymes decreased to very low levels between the 2nd and 4th days of germination and thereafter, by comparison, showed very little further diminution (Table 6).

The phosphatases showed patterns of change during germination that were distinctly different from those for the cytochrome C reductases (Table 7). For young cotyledon tissue glucose-6-phosphatase was substantially more active than 5′-nucleotidase in both rough and smooth microsomes. However, in sharp contrast to the behavior of cytochrome C reductase, both phosphatases

Table 5. Changes during germination in phosphatase specific activities of smooth and rough microsomal membranes

Tissue age (days)	Glucose-6-phosphatase		5′-Nucleotidase	
	Smooth microsomes	Rough microsomes	Smooth microsomes	Rough microsomes
2	0·14 ± 0·07	0·10 ± 0·02	0·06 ± 0·01	0·06 ± 0·01
4	1·08 ± 0·21	1·72 ± 0·51	0·23 ± 0·04	0·23 ± 0·03
7	0·32 ± 0·05	0·51 ± 0·09	0·25 ± 0·04	0·18 ± 0·03
9	0·42 ± 0·13	0·15 ± 0·03	0·34 ± 0·11	0·13 ± 0·04

Activities are expressed as μg P released per μg phospholipid P per hr. Standard errors are indicated; $n = 3$–5.

Table 6. Changes during germination in total cytochrome C reductase activities of smooth and rough microsomal membranes

Tissue age (days)	NADH–cytochrome C reductase		NADPH–cytochrome C reductase	
	Smooth microsomes	Rough microsomes	Smooth microsomes	Rough microsomes
2	265 ± 48	56·9 ± 15·5	25·4 ± 4·9	3·6 ± 1·2
4	181 ± 36	9·2 ± 3·3	2·1 ± 0·3	0·61 ± 0·15
7	31·7 ± 10·6	4·6 ± 1·0	0·39 ± 0·05	0·29 ± 0·03
9	8·8 ± 1·9	3·2 ± 0·7	0·37 ± 0·07	0·23 ± 0·01

Activities are expressed as μmol reduced cytochrome C per 20 g of cotyledon tissue per hr. Standard errors are indicated; $n = 3$ or 4.

increased in activity to reach a peak by the 4th day of germination before declining. By the time the tissue was senescent glucose-6-phosphatase had declined to a very low activity in both rough and smooth membranes by comparison with its peak activity at day 4. Yet, even by the ninth day of germination 5′-nucleotidase had decreased to only two thirds of its peak activity for smooth membrane and half its peak for the rough microsomes (Table 7).

Phosphatase activities were recoverable within acceptable limits in the various fractions arising from the isolation procedure. Recoveries for each enzyme ranged from 85 to 110% of homogenate activity regardless of tissue age. This was not the case for the cytochrome C reductases. Typically, NADH–cytochrome C reductase was about 65% recoverable with respect to homogenate and NADPH–cytochrome C reductase only 20% recoverable. This loss of cytochrome C reductase activity has been observed in other tissues as well and has been attributed to inactivation of the enzymes by dilution and pelleting [14]. However, in the present study the recoveries for these enzymes, despite being low, were very consistent for all ages of tissue examined and the total activities in the microsomal fractions expressed on the basis of tissue weight were reproducible within acceptable limits (Tables 6 and 7).

To determine whether attenuation of microsomal enzyme activity signified actual breakdown of membranes as distinct from inactivation of the enzymes on otherwise intact membranes, total levels of phospholipid-P in the microsomal subfractions were determined at various ages. The data illustrated in Table 3 indicate unequivocally that microsomal phospholipid does indeed decrease as germination progresses. This diminution presumably reflects actual breakdown of cytoplasmic membranes in the cells. Both rough and smooth subfractions show the greatest losses between days 2 and 4, although the smooth membrane deteriorates much more rapidly than the rough during this period (Table 3).

Changes in enzyme sp. act. in the microsomal subfractions were monitored and these data are expressed as a function of phospholipid (Tables 4 and 5). For smooth microsomes NADPH–cytochrome C reductase declined progressively with age, although it lost by far the major proportion of its sp. act. between days 2 and 4 (Table 4). By contrast NADH–cytochrome C reductase showed an increase in sp. act. between the second and fourth days of germination despite a decline in total activity (compare Tables 4 and 6). Thereafter its sp. act. rapidly declines to reach a low level by day 7 and lower still by day 9 (Table 4). For the rough microsomal fraction the activi-

Table 7. Changes during germination in total phosphatase activities of smooth and rough microsomal membranes

Tissue age (days)	Glucose-6-phosphatase		5′-Nucleotidase	
	Smooth microsomes	Rough microsomes	Smooth microsomes	Rough microsomes
2	114 ± 60	37·5 ± 8·7	49·0 ± 12·8	23·4 ± 6·5
4	365 ± 69	322 ± 96	78·7 ± 16·6	43·7 ± 5·4
7	74·3 ± 10·8	69·6 ± 7·0	58·9 ± 9·4	23·9 ± 1·0
9	56·9 ± 17·2	20·3 ± 1·8	45·6 ± 15·0	18·2 ± 5·7

Activities are expressed as μg P released per 20 g of cotyledon tissue per hr. Standard errors are indicated; $n = 3$–5.

ties of both cytochrome C reductases decreased progressively and more or less in parallel, showing their steepest decline between days 2 and 4 (Table 4).

Patterns of change in phosphatase sp. act. were more erratic than those for cytochrome C reductase. For smooth microsomes glucose-6-phosphatase initially showed an increase in sp. act., reaching a peak by the 4th day of germination; subsequently, it declined rapidly (Table 5). 5′-Nucleotidase also increased in sp. act. between days 2 and 4, but thereafter remained high and in fact showed a slight further increase between days 7 and 9 (Table 5). In the rough microsomal fractions both phosphatases showed increases in their sp. act. between days 2 and 4, but then declined. Glucose-6-phosphatase decreased more sharply, particularly between days 4 and 7, than did 5′-nucleotidase (Table 5).

DISCUSSION

Comparisons of total phospholipid in the microsomal subfractions for 2-day-old tissue revealed that the smooth subfraction contains more than twice as much membrane as the rough. This presumably reflects the relative abundance of smooth and rough surfaced membranes in the storage cells of the cotyledons at this stage of germination. The procedure used to subfractionate microsomes is one that has previously been shown to be effective for several tissues [15–18]. The conclusion that a definitive separation was achieved in this study is supported by the different RNA-P:phospholipid-P ratios obtained for the two subfractions, in particular the very low ratios for smooth microsomes, and a discrete partitioning of biochemically distinct membranes between the two subfractions. For example, prior to the onset of senescence cytochrome C reductase sp. act. were consistently 2–3 times higher for smooth microsomal fractions than for those containing rough microsomes. Higher concentrations of cytochrome C reductase activity on smooth microsomes than on corresponding rough surfaced membrane have also been reported for other systems [17–19]. In a previous study of cytoplasmic membrane senescence in cotyledons of *P. vulgaris*, which dealt exclusively with the cytochrome C reductases of smooth microsomes, sp. act. were determined conventionally using protein as a base [11]. However, in the present study sp. act. were determined as a function of phospholipid rather than protein in order to minimize spurious contributions by ribosomal protein and any soluble protein trapped inside the membrane vesicles.

As germination progresses, levels of phospholipid in both rough and smooth microsomal fractions decrease more or less in parallel. It seems logical to interpret this as reflecting deterioration and ultimate breakdown of cytoplasmic membranes in the cells as the pressures of senescence become more intense. In fact the very pronounced drop in microsomal phospholipid levels between the 2nd and 4th days of germination when compared with a more gradual decline during the later stages of germination (Table 3) suggests that breakdown of cytoplasmic membranes is an early event in the temporal sequence of cytoplasmic deterioration. Again, loss of phospholipid rather than protein was monitored because of the inherent difficulties in measuring membrane protein exclusively in these fractions.

The profiles of phospholipid attenuation indicate that rough and smooth surfaced cytoplasmic membranes break down in approximate synchrony as germination progresses. However, comparisons of profiles illustrating changes in microsomal enzyme activities during the same period suggest that functionally distinct regions within these two morphological types of membrane have differing sensitivities to senescence. For example, total NADPH–cytochrome C reductase activity of smooth microsomes drops off much more rapidly on a percentage basis between days 2 and 4 than does NADH–cytochrome C reductase (Table 6). Since these reductions correlate with a corresponding decrease in total phospholipid of the fraction (cf. Tables 3 and 6), they can be reasonably interpreted as reflecting deterioration of those membranes bearing the enzyme activities, although molecular disaggregation of other membranes not possessing the enzymes is not precluded. However, the sharper decline of NADPH–cytochrome C reductase suggests that the two enzymes are associated with different regions of membrane and that regions bearing NADPH–cytochrome C reductase are more sensitive to the

pressures of senescence than those possessing NADH–cytochrome C reductase.

This interpretation is further borne out by the profiles illustrating changes in the sp. act. of the enzymes. Alterations in sp. act. can provide information about the temporal relationship between loss of enzyme activity and actual breakdown of membrane structure. If, for example, loss of enzyme activity and a dismantling of membrane structure are simultaneous events there should be no change in enzyme sp. act. for that membrane remaining. Alternatively, if enzymes are being inactivated on otherwise intact membrane, sp. act. should decrease. Finally, if other membranes, isolated with the fraction being analyzed but not bearing the enzymes being monitored, are being broken down selectively or more rapidly than those membranes possessing the enzymes, the sp. act. of the enzymes should increase. For the system under study, the sp. act. for NADPH–cytochrome C reductase in the smooth microsomal fractions decreased between days 2 and 4 of germination, yet that for NADH–cytochrome C reductase increased during the same period (Table 4). This strongly indicates that the enzymes are associated with distinct regions of the membrane. Indeed it has been previously demonstrated for mammalian tissue that both rough and smooth microsomal membranes are markedly heterogeneous with respect to distribution of enzymes [15]. The decline in NADPH–cytochrome C reductase sp. act. indicates that inactivation of the enzyme preceded and occurred more quickly than breakdown of cytoplasmic membranes in general. The increased sp. act. of NADH–cytochrome C reductase during the same period indicates that cytoplasmic membranes not possessing the enzyme are undergoing deterioration more rapidly than those which do possess it. This strongly supports the premise that different types of cytoplasmic membranes, or perhaps functionally distinct regions within the same type, display differential sensitivities to senescence. By contrast the approximately parallel decline in sp. act. of the cytochrome C reductases in rough microsomes suggests that regions of the rough endoplasmic reticulum bearing these enzymes are senescing in approximate synchrony.

Further evidence for distinct patterns of cytoplasmic membrane senescence comes from an analysis of microsomal phosphatases, for the behavior of these enzymes during cytoplasmic senescence proved to be quite different from that of the cytochrome C reductases. In particular the total phosphatase activities of both rough and smooth microsomal fractions increased between the 2nd and 4th days of germination (Table 7). Yet corresponding cytochrome C reductase activities decreased during the same period (Table 6). These observations can logically be interpreted as indicating that microsomal phosphatases and cytochrome C reductases are associated with different membranes, or different regions of the same membrane, which are independently influenced by senescence. In fact the increasing levels of total phosphatase activity in the microsomal fractions between days 2 and 4 and corresponding increases in their sp. act. suggest either synthesis of new cytoplasmic membrane during this period or a refurbishing of pre-existing membrane. Yet other cytoplasmic membranes are clearly undergoing autolysis during the same period as indicated by the pronounced drop in phospholipid levels of the isolated microsomal subfractions (Table 3). The persistently high sp. act. for 5′-nucleotidase of smooth microsomes by even the ninth day of germination relative to its activity at earlier stages (Table 5) suggests that membrane bearing this enzyme is particularly resistant to autolysis by comparison with other cytoplasmic membranes.

It does, therefore, seem reasonable to suggest that the breakdown of cytoplasmic membranes in cotyledon tissue during germination is finely regulated in the sense that functionally distinct regions of membrane, recognizable by the enzymes they possess, display symptoms of senescence in distinguishable temporal patterns. These changes do not appear to be influenced by the presence or absence of ribosomes on the membranes. However for each enzyme the proportions of total microsomal activity partitioning between the rough and smooth subfractions showed some variation. This indicates that corresponding regions of rough and smooth membrane do not undergo senescence in complete synchrony. The mechanism by which cytoplasmic membranes are able to achieve differential sensitivity to senescence is not clear, but it may involve focal autolysis, a phenomenon well documented for certain mammalian systems [20].

EXPERIMENTAL

Biochemicals required for the enzyme assays were obtained from Sigma. Untreated seeds of *P. vulgaris* were germinated in the dark at 29° in moist vermiculite. Cotyledons were harvested at specified intervals during the first 10 days of germination.

Cytoplasmic membrane isolation. Rough and smooth microsomal fractions were prepared from 2, 4, 7 and 9-day-old cotyledons using the basic procedure of Dallner and Ernster [15] as modified for plant tissue in Ref. [11]. Cotyledon tissue (20 g) was homogenized with a mortar and pestle in 75 ml of 0·3 M sucrose–0·05 M $NaHCO_3$, pH 7·5. The homogenate was filtered through 4 layers of cheesecloth and the filtrate then centrifuged at 10000*g* for 20 min. The resulting supernatant was made 15 mM with CsCl and centrifuged again through a barrier of 1·3 M sucrose–15 mM CsCl at 122000*g* for 210 min in a Spinco Type 50 fixed angle rotor. The smooth microsomes collected at the interface and the rough microsomes formed a pellet at the bottom of the tube. Both fractions were removed, diluted at least 3-fold with 0·05 M $NaHCO_3$, pH 7·5, and pelleted by centrifugation at 122000*g* for 60 min. The resulting pellets were resuspended in from 2 to 4 ml of 0·05 M $NaHCO_3$, pH 7·5, and stored at −20° until required for assays.

Assay procedures. Rotenone-insensitive NADH–cytochrome C reductase and NADPH–cytochrome C reductase were assayed as described previously [11]. Glucose-6-phosphatase and 5′-nucleotidase were also measured as described previously [7] except that in the 5′-nucleotidase assay the reaction time was increased from 15 min to 1 hr. Levels of phospholipid-P and ribonucleic acid-P in the isolated fractions were determined according to the procedures of Dallner *et al.* [21] and Schneider [22], respectively.

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