# Ascorbic acid requirement for increased peroxidase activity during potato tuber slice aging

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The increase in peroxidase activity during aging is a well known process depending on de novo enzyme synthesis. The enhancement of peroxidase is strongly inhibited by lycorine, an ascorbic acid biosynthesis inhibitor. The inhibitory effect of lycorine can be abolished by experimentally increasing the ascorbic acid concentration in the tissues. Conversely, glucose-6-phosphate dehydrogenase enhancement – which also occurs during the aging of potato slices – depends on protein synthesis but does not require ascorbic acid. It is suggested that the role of ascorbic acid in the development of peroxidase activity may be related to controlling the synthesis of hydroxyproline-containing proteins. A possible relationship between peroxidase increase and hydroxyproline-containing proteins biosynthesis is discussed.

*Ascorbic acid* *Peroxidase increase* *Aging* *Lycorine* *3,4-Dehydroproline*

## 1. INTRODUCTION

Our previous studies showed that ascorbic acid (AA) is widely utilized in cell metabolism [1]; AA is needed for the 'in vivo' synthesis of hydroxyproline-containing proteins [2]; AA is specifically utilized for the development of KCN-resistant respiration [3]; AA is required for cell division [4].

These results were obtained by employing lycorine – an alkaloid that inhibits in vivo AA biosynthesis. Lycorine has proved to be a useful tool for studying AA-dependent metabolic reactions in AA-synthesizing cells [1].

Since during aerobic incubation of storage tissue slices (aging) AA is metabolized at fast rates, here we examined whether a relationship exists between AA metabolism and the increase in certain enzymatic activities occurring during aging. Among the enzymes which are enhanced after slicing storage tissue [5,6], peroxidase and glucose-6-phosphate dehydrogenase have been considered.

A remarkable increase in peroxidase activity following wounding has been observed [7] and it has been shown that the total peroxidase activity in wounded potato tissue represents the sum of the activities of several isoperoxidases, which constitute specific markers of wound-induced cell differentiation [8].

The relationship between AA concentration and development of peroxidase activity, ignoring for the moment questions of characterization or function of peroxidase isoenzymes, has been investigated.

## 2. MATERIALS AND METHODS

1 mm thick potato tuber slices (*Solanum tuberosum*, 1984 harvest) were repeatedly washed in tap water and used immediately after cutting ('fresh slices') or after incubation in water at 20°C for 24 h on a shaker ('aged slices'). Lycorine, cycloheximide, puromycin, 3,4-dihydroproline and AA were added where indicated; compounds were from: Istituto di Chimica Biologica, Università di Napoli, Italia (lycorine); Sigma, St. Louis, USA (AA, cycloheximide, puromycin, 3,4-dehydroproline, bovine serum albumin, cysteine, NADP and glucose 6-phosphate); Carlo Erba,

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Milano, Italia (mannitol, EDTA, $MgCl_2$, $H_2O_2$ and guaiacol).

Total peroxidase activity was tested in mitochondrial and microsomal fractions and in the cytosol.

A 50 g sample of slices was homogenized in a Waring blender for 60 s with 2 vols (w/v) of cold buffer containing 50 mM Tris-HCl pH 8.0, 0.3 M mannitol, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% bovine serum albumin and 0.05% cysteine. The homogenate was strained through nylon cloth and centrifuged at 600 × *g* for 10 min to remove cell debris and nuclei.

Mitochondria were precipitated at 10000 × *g* for 15 min, washed, and suspended in a wash medium (pH 7.2) similar to the grinding medium, lacking EDTA and cysteine. Then they were centrifuged for 15 min at 6000 × *g* and resuspended in wash medium [9]. The mitochondrial fraction was sonicated with a Branson sonifier (model W 18 SD) for 1 min at intervals of 20 s at 50 W and 0°C.

The microsomal fraction was precipitated by centrifugation at 100000 × *g* for 1 h and resuspended in the same medium as the mitochondria [10].

Peroxidase activity was assayed by measuring absorbance at 470 nm using guaiacol and hydrogen peroxide according to Chance and Maehly [11]. The activity was expressed as units (1 unit = $\Delta A_{470}/0.100$ min per mg protein).

Glucose-6-phosphate dehydrogenase activity was tested by measuring the rate of NADP reduction at 340 nm [12].

AA was assayed as described in [4] and protein according to Lowry et al. [13].

## 3. RESULTS

Table 1 shows that peroxidase and glucose-6-phosphate dehydrogenase activities are enhanced by a factor of 11 and 2.5, respectively, during the aging of potato tuber slices.

Slicing-induced increase in peroxidase activity occurs in the mitochondrial and microsomal fractions as well as in cytosol; the highest increase (22-fold) was observed in microsomes (fig.1).

Peroxidase activity increase seems to be due to protein synthesis. Our experiments confirm the results in [14,15], that puromycin and cycloheximide prevent the rise in peroxidase activity (fig.2).

Table 1

Increase in peroxidase and glucose-6-phosphate dehydrogenase activities during aging of potato slices

| Sample | Peroxidase units[a] | G-6-PDH[b] |
|---|---|---|
| Fresh slices | 4.4 | 67 |
| 24 h aged slices | 49.1 | 166 |

[a] 1 unit = $\Delta A_{470}/0.10$ min per mg protein
[b] nmol NADP reduced/min per mg protein

The values indicate activities observed in the cytosol

To determine whether AA is involved in the enhancement of peroxidase, we tested the influence of lycorine, a well known AA biosynthesis inhibitor. The data in table 2 show that AA content rises during aging and that the addition of 10 $\mu$M lycorine inhibits AA biosynthesis by 86%. Complete inhibition is obtained at 30 $\mu$M lycorine (not shown). Treatment with lycorine during aging strongly inhibited the development of peroxidase activity (fig.2). The degree of inhibition by the

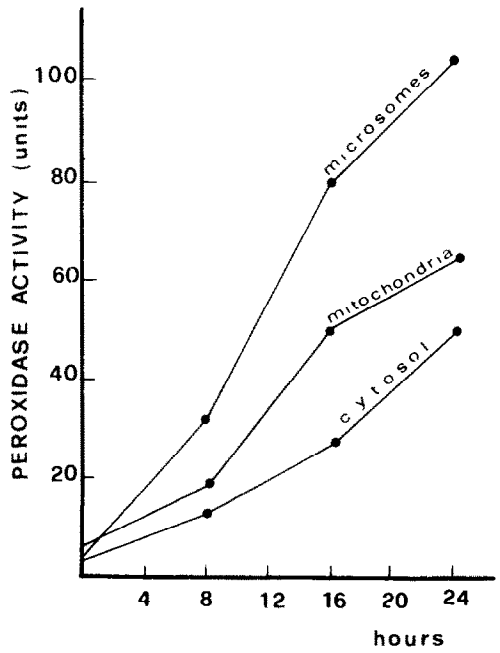


Fig.1. Time course of peroxidase activity during aging of potato tuber slices.

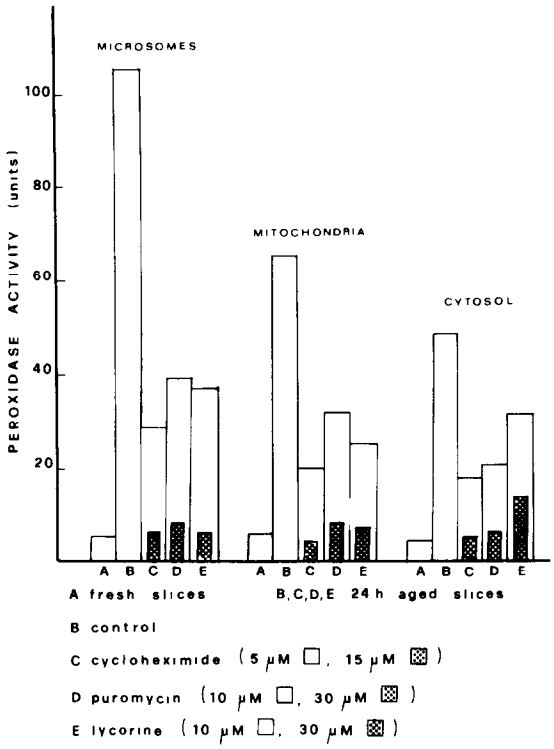


Fig.2. Effect of cycloheximide, puromycin and lycorine on the development of peroxidase activity in aged potato slices.

alkaloid was found to be highest in the microsomal and mitochondrial fractions (30 $\mu$M lycorine inhibited 93 and 88%, respectively) and slightly lower in the soluble fraction (71%).

Table 2

Effect of lycorine and cycloheximide on ascorbic acid synthesis of potato slices

| Sample | AA content | AA synthesized ($\mu$g/g dry wt) | Inhibition (%) |
|---|---|---|---|
| Fresh slices | 420 | | |
| Aged | 900 | 480 | |
| Lycorine aged | 488 | 68 | 86 |
| Cycloheximide aged | 862 | 442 | 8 |

Potato slices were incubated for 24 h in 10 $\mu$M lycorine or 5 $\mu$M cycloheximide

These data suggest that inhibition of peroxidase activity increase is in some way related to the decrease in AA. To show that the rise of peroxidase activity is indeed dependent on the amount of AA present in the cell, we carried out experiments demonstrating that the inhibiting effect produced by lycorine is prevented by exogenous AA addition. The 64% inhibition induced by 10 $\mu$M lycorine was completely prevented when 1 mM AA was added to slices during aging (table 3); AA addition was ineffective in the cycloheximide inhibitory effect (table 3).

Further indirect information about the existence of a relationship between AA and the increase in peroxidase activity during aging has been obtained by using 3,4-dehydroproline. Recently it has been shown that this proline analog is an active in vivo inhibitor of peptidylprolyl hydroxylase [16], the enzyme involved in the synthesis of hydroxyproline-containing proteins. The treatment of the slices with 3,4-dehydroproline strongly inhibited the rise in peroxidase activity and at the same time led to an increase in AA content with respect to untreated slices (table 4). Dehydroproline had no effect on peroxidase activity in vitro.

In marked contrast to the rise in peroxidase activity, glucose-6-phosphate dehydrogenase increase following wounding of potato tuber tissue

Table 3

Ascorbic acid prevention of lycorine effects on microsomal peroxidase development during aging of potato slices

| Sample | Peroxidase units[a] | Inhibition (%) |
|---|---|---|
| Fresh slices | 4.7 | |
| Aged | 105 | |
| Lycorine aged | 39.5 | 64 |
| Lycorine + AA aged | 100 | |
| Cycloheximide aged | 30 | 75 |
| Cycloheximide + AA aged | 31.5 | 73 |
| Ascorbic acid aged | 107 | |

[a] 1 unit = $\Delta A_{470}/0.10$ min per mg protein

Potato slices were incubated for 24 h in all treatments: lycorine (final concentration 10 $\mu$M), cycloheximide (final concentration 5 $\mu$M) and AA (final concentration 1 mM) were added as indicated

Table 4

Effect of 3,4-dehydroproline on ascorbic acid content and on the increase in peroxidase and on G-6-PDH activities during aging of potato tuber slices

| Sample | Peroxidase units[a] | G-6-PDH[b] | AA content[c] |
|---|---|---|---|
| Fresh slices | 3.9 | 70 | 420 |
| Aged | 46.8 | 168 | 900 |
| Dehydroproline aged | 24 | 177 | 1096 |

[a] 1 unit = $\Delta A_{470}/0.10$ min per mg protein
[b] nmol NADP reduced/min per mg protein
[c] $\mu$g/g dry wt

Potato slices were incubated for 24 h in 0.1 mM 3,4-dehydroproline; peroxidase and G-6-PDH activities were determined in the cytosol

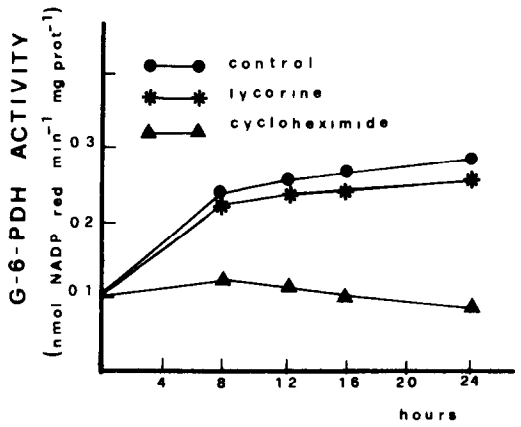


Fig.3. Effect of lycorine and cycloheximide on the increase of glucose-6-phosphate dehydrogenase activity during aging of potato slices.

was affected by neither 3,4-dehydroproline (table 4) nor lycorine, whereas it was completely inhibited by cycloheximide (fig.3).

## 4. DISCUSSION

Our results confirm that the increase of peroxidase activity occurring during aging depends on protein synthesis and also requires the presence of ascorbic acid. By inhibiting AA biosynthesis with lycorine, peroxidase increase is strongly inhibited. When the endogenous concentration of AA is artificially increased, the inhibitory effect of lycorine on peroxidase enhancement is completely abolished.

Moreover, although AA is present in the cell, the increase of peroxidase activity is also prevented by inhibiting prolyl hydroxylase activity with 3,4-dehydroproline.

Therefore we might argue that peroxidase enhancement is a process involving both AA and the activity of prolyl hydroxylase, i.e., the biosynthesis of hydroxyproline-containing proteins. It has already been proved that: (i) AA is needed for the in vivo synthesis of 'hypro'-proteins [2] and (ii) in the presence of 3,4-dehydroproline, which inhibits the peptidylproline hydroxylation, the hypro-proteins synthesized in great quantities during aging are strongly underhydroxylated and therefore underglycosylated [16].

The data above become more interesting if the effects induced by lycorine, 3,4-dehydroproline and cycloheximide on the increase in glucose-6-phosphate dehydrogenase are considered. The enhancement in enzyme activity during aging is very likely due to de novo synthesis since it is completely stopped by cycloheximide, but it is affected by neither lycorine nor 3,4-dehydroproline. This indicates that, unlike peroxidase increase, the rise in G-6-PDH does not require AA or peptidylproline hydroxylase activity.

It should be noted that 3,4-dehydroproline has been proved to have little effect on nitrate reductase induction by nitrate, on wound-induced increase in amino acid uptake and on protein synthesis [16]. Since 3,4-dehydroproline does inhibit the increase in peroxidase, the involvement of peptidyl hydroxylase activity, together with that of AA, in the process suggests that the peroxidase rise may be related to the synthesis of hydroxyproline-containing proteins.

At present we cannot explain the link existing at the molecular level between the synthesis of 'hypro'-proteins and the rise in peroxidase during aging. We are now looking for either some peroxidases containing hydroxyproline in their polypeptide chain or some histones involved in the control of peroxidase genes which could be hydroxylated during aging.

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