ASCORBIC ACID REQUIREMENT FOR BIOSYNTHESIS OF HYDROXYPROLINE-CONTAINING PROTEINS IN PLANTS

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1. Introduction

The concentration of ascorbic acid (AA) in the plant cell is usually high and seems to be in excess of the amount involved in the respiratory processes [1,2]. Recently, we have demonstrated that a large amount of ascorbic acid is utilized in the cell metabolism of higher plants [3]. We have suggested that the intensive AA consumption in the cells could be related to its eventual involvement in the synthesis of hydroxyproline-containing proteins [4].

Since during the aerobic incubation of slices from storage tissues (aging) a remarkable synthesis of hydroxyproline-containing proteins develops [5–7], we used this system to study the relationship between AA and the biosynthesis of hydroxyproline-containing proteins.

2. Materials and methods

Cylinders of potato tuber (*Solanum tuberosum*) tissue, 0.9 cm in diameter, were removed with a cork borer and sliced on a sliding microtome. The slices, 1 mm thick, were washed repeatedly in tap water and used immediately ('fresh' slices), or after incubation in water at 20°C for 8–36 h, with air bubbled through the medium ('aged' slices). Lycorine was added where indicated.

Two different lots of potato tubers were used for the experiments: tubers maintained at 4°C for at least 4 months after harvesting ('stored' potatoes) and freshly harvested tubers.

For the assay of hydroxyproline, the slices (7–8 g fresh wt) were extracted 6 times with 10 ml boiling 80% ethanol, washed with anhydrous ethanol and dried at 90°C. This material, which contained both cytoplasmic and cell wall proteins, but did not contain free amino acids, was hydrolysed with 6 N HCl at 110°C for 18 h. Hydroxyproline contents were determined by the colorimetric procedure of Firshein and Shill [8], with *p*-dimethylaminobenzaldehyde as a reactant. Hydroxyproline was also identified chromatographically by using the procedure of Prockop et al. [9].

3. Results

Table 1 shows that the protein-hydroxyproline content of stored potato tuber slices was greatly enhanced during aerobic incubation; the total amount increased from 86–302 μg/g dry wt in 36 h.

Table 1
Increase in protein-hydroxyproline content during the aging of stored potato tuber slices[a]

Incubation time (h)	Hypro[b] (μg/g dry wt)
0	86
8	125
16	212
24	275
36	302

[a]Incubation of slices freshly cut from storage tissues is accompanied by a number of physiological and biochemical changes collectively called 'aging' [10]
[b]Hypro, protein-hydroxyproline

Table 2
Effect of lycorine on the biosynthesis of protein-hydroxyproline during the aging of stored potato tuber slices[a]

	Treatment	Hypro[b] content (μg/g dry wt)	Hypro[b] synthesized in 36 h	% Inhibition
Fresh slices		90		
Aged slices	H_2O	283	193	
Aged slices	Lycorine 1 μM	233	143	26
Aged slices	Lycorine 3 μM	171	81	58
Aged slices	Lycorine 10 μM	130	40	79

[a]Potato slices were aged for 36 h
[b]Hypro, protein-hydroxyproline

To determine whether the biosynthesis of hydroxyproline-proteins is correlated to the endogenous level of ascorbic acid, we have worked with two different systems: first, we used lycorine, a compound inhibiting specifically the biosynthesis of AA during aging [3]; second, we used two different lots of potato tubers, i.e., stored and freshly-harvested potatoes, respectively, which are characterized by different ascorbic acid contents [4].

The addition of lycorine during the aging of stored potato tuber slices strongly inhibited the biosynthesis of protein-hydroxyproline. Data in table 2 show that 1 μM lycorine inhibited the biosynthesis by 26% and 10 μM lycorine by as much as 80%.

When slices from freshly-harvested potato tubers were used, the level of protein-hydroxyproline synthesized was twice as high as with the stored slices. Starting with 95 μg protein-hydroxyproline/g dry wt of 'fresh' slices (a value similar to that found in stored potato tubers), the protein-hydroxyproline content increased to 580 μg/g dry wt in slices 'aged' for 36 h. Furthermore, the addition of lycorine during the aging of the slices did not affect the increase in protein-hydroxyproline content. The strong rise in the protein-hydroxyproline content of the slices from freshly-harvested potato tubers and their low sensitivity to lycorine are probably due to their high level of ascorbic acid. In fact, freshly-harvested potatoes contain much more ascorbic acid (4–5-fold) than stored ones and lycorine, while inhibiting AA biosynthesis, does not interfere with AA utilization [4,11]. These data clearly suggest that the increase in protein-hydroxyproline content is related to the amount of ascorbic acid present in the cell. Further support for this conclusion was obtained by demonstrating that the lycorine-inhibition in slices of stored tubers is prevented by the administration of AA. The 65% inhibition induced by 3 μM lycorine was almost completely prevented when 1 mM AA was added to the slices during the aging process (table 3).

Table 3
Prevention of the effect of lycorine on protein-hydroxyproline biosynthesis by ascorbic acid during the aging of stored potato tuber slices[a]

	Treatment	Hypro[b] content (μg/g dry wt)	Hypro synthesized in 36 h	% Inhibition
Fresh slices		95		
Aged slices	H_2O	291	196	
Aged slices	Lycorine 3 μM	163	68	65
Aged slices	Lycorine 3 μM + AA 1 mM	264	169	14
Aged slices	AA 1 mM	312	217	

[a]Potato slices were aged for 36 h – to avoid AA exhaustion, the medium was changed every 12 h
[b]Hypro, protein-hydroxyproline

4. Discussion and conclusion

Experiments in vivo and in vitro suggest that in plants [12–14], like in animals [15–17], hydroxyproline-containing proteins are synthesized at the ribosomal level as ordinary proline-containing polypeptide chains and that, subsequently, some of the proline residues present in the chain are hydroxylated by means of a prolyl-hydroxylase, similar to that isolated from cells which synthesize and secrete collagen [18]. Ascorbic acid has been regarded for many years as a highly specific cofactor in the hydroxylation of collagen peptidyl-proline [19,20] but Rhoads et al. [21] and Hobza [22] recently argued that the role of AA is less specific than it was originally thought. In fact, their studies in vitro have shown that prolyl-hydroxylase can function in the complete absence of ascorbic acid if other non-physiological reducing agents, like dithiothreitol, are added to the system. The results of the present study which show, for the first time, that AA is required to carry out the in vivo synthesis of hydroxyproline-containing proteins in plants, are in accordance with the original views of Robertson [19] and Stone and Meister [20] who considered AA to be a specific factor in proline hydroxylation.

In the light of these results, the intensive consumption of ascorbic acid during plant cell metabolism [3] can be explained, since hydroxyproline-containing proteins, both structural and enzymatic, are widespread in the plant cell [23–25]. With regard to the possible physiological implications of AA requirements, at least two biological processes should be emphasized. One is represented by plant growth. Lamport [26], Cleland [27] and Ridge and Osborne [28] have shown that expansion growth is dependent on the biosynthesis of hydroxyproline-rich proteins in the cell wall. Srivastava [29], on the other hand, has reported that nuclear histones containing hydroxyproline are synthesized during cell division in tobacco callus and we have demonstrated that, in the absence of AA, the cell cycle is blocked in the interphase stage [30]. Since plant growth depends on both cell division and cell expansion, it appears likely that ascorbic acid is involved in plant growth regulation through the control of the biosynthesis of hydroxyproline-containing proteins. In this connection, our data appear to provide an explanation for the often observed correlations between ascorbic acid, auxin and growth in plants [31,32].

Another biological process involving massive AA utilization, and synthesis of hydroxyproline-containing proteins, is represented by some of the biological defence mechanisms in both plants [33] and animals [34]. We are now studying this aspect of the problem.

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