Inhibition of protease activity by polyamines

Relevance for control of leaf senescence

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1. INTRODUCTION

The polyamines spermidine and spermine and the related diamines putrescine and cadaverine are polycations which are synthesized in most living cells [1–3] and have been implicated as essential growth factors for plants [4,5]. Polyamines applied exogenously are potent inhibitors of senescence of oat leaf protoplasts [6,7] and of leaves and storage tissue from several plants [8,9]. We have also reported that polyamine–biosynthetic enzyme activities and titer decrease in senescing attached and detached oat leaves incubated in the dark [10], and in potato tubers during dormancy [11]. When growth of pea buds and internodes is stimulated or inhibited by phytochrome conversion [12,13] or hormonal treatment [14], polyamine synthesis and titer show rapid and parallel changes. These observations, together with the demonstrated roles of polyamines in cell proliferation suggest that they are involved in processes which control plant growth and senescence.

One of the early events in leaf senescence is the well-documented rise in protease activity [15]. However, little is known about the regulation of proteolysis itself during leaf senescence. Considerable evidence has accumulated which shows that involvement of polyamines in cellular activity may be mediated via interaction with macromolecules [16]. Since exogenous application of polyamines retards senescence and the rise in protease activity is one of the early events in senescence, we reasoned that these compounds may in fact decrease senescence through inhibition of protease activity. We therefore undertook to investigate the effects of various naturally-occurring polyamines on protease activity in connection with their action in controlling senescence of excised oat leaves.

We now report that application of exogenous polyamines to leaf segments retards senescence by decreasing protease activity and chlorophyll loss. Furthermore, we provide evidence that the decrease in protease activity occurs by binding of the polyamines to the enzyme.

2. MATERIALS AND METHODS

2.1. *Plant material*

Oat seeds (*Avena sativa*, cv. Victory) obtained from Swedish Seed Co. (Svalov) were grown in controlled growth rooms [10]. The first leaf of two-week-old seedling was used.

2.2. *Conditions of treatments*

For in vivo effects of polyamines, excised leaves were sterilized as in [10] and the lower epidermis removed by stripping with fine forceps. Four such median (4.5 cm) leaf segments were floated, stripped side down in Petri dishes on 5 ml of either 1 mM phosphate buffer (pH 5.8), or on buffered solution containing 1 mM of various polyamines (HCl salt, Sigma). Polyamines at 1 mM were op-

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timum for reducing chlorophyll breakdown [8]. We also found that spermidine treatment for 48 h is most effective in preventing the rise of protease activity and chlorophyll breakdown with no visible toxic effects (not shown). Therefore, 1 mM spermidine was used for most of these experiments. Spermine was most effective in 1 h treatments, but it produced toxic effects when used for 48 h. After incubation for 48 h in the dark, the leaves were thoroughly washed to remove excess polyamines and used for measurements of chlorophyll content and protease activity. All manipulations were performed under aseptic conditions in a laminar flow hood. For in vitro effects of polyamines on protease activity, various concentrations of polyamines were added to the crude enzyme extract at time of assay.

2.3. *Enzyme extraction*

Fifteen leaf segments (4.5 cm each) were homogenized in a pre-chilled mortar and pestle with 2 ml cold 50 mM phosphate–citrate buffer, (pH 6.0). The homogenates were centrifuged at 12 000 × *g* for 15 min at 4°C. The resulting clear supernatant fractions were assayed for protease activity.

2.4. *Enzyme assay*

Protease activity was measured by using Azocoll (Calbiochem) as the substrate. In 1.0 ml a final vol.; the reaction mixture contained 5 mg Azocoll, 0.8 ml 50 mM phosphate–citrate buffer (pH 4.2) for acid protease and pH 6.6 for neutral protease, and 0.2 ml crude enzyme. For in vitro effects, the reaction mixture included 0.1 ml various concentrations of polyamines. The tubes were stoppered, vortexed and floated in a water bath equipped with a shaker and maintained at 43°C for 3 h. These conditions were found to be optimum for both the acid and neutral protease activities. Blanks were prepared similarly, either with boiled enzyme or no enzyme. The reaction was terminated by immersing the tubes in an ice bath and subsequently centrifuging to remove the undigested Azocoll. The optical density of the supernatants was measured at 520 nm. Enzyme activity was linear with time and with concentration of crude extract. Dialysis of the enzyme extract did not alter its activity. Enzyme activity is expressed as units . mg protein^{-1} . h^{-1}. One unit of activity is the amount of enzyme which produces a ΔA of 0.1 in 1 h. Each assay was replicated 3–4 times and each experiment was repeated at least 2 times.

2.5. *Chlorophyll content*

Total chlorophyll was measured spectrophotometrically in hot 80% ethanol leaf extracts by reading the absorbance at 665 nm.

2.6. *Protein content*

Soluble protein content in the enzyme extracts was measured as in [17] using bovine serum albumin as a standard.

2.7. *Analysis of results*

Initial values of protease activity and chlorophyll content were measured on leaf samples at the start of each experiment. The results are expressed as units of activity or as percentage of the initial values. The data presented are from single experiments which are representative of 2–6 experiments of each treatment.

3. RESULTS

3.1. *In vivo inhibition of protease activity*

When excised oat leaves are incubated in the dark, their protease activity increases dramatically (table 1). Increased activity was observed in two

Table 1

In vivo inhibition of protease activity and chlorophyll loss in oat leaves by 1 mM polyamines and diamines

Treatment	Protease activity (units . mg protein^{-1} . h^{-1} ± SE)		Chlorophyll content
	Acid	Neutral	(% loss)
Control			
initial	0.8 ± 0.04	1.7 ± 0.08	0
final	5.2 ± 0.11 (0)	4.0 ± 0.19 (0)	73
Cadaverine	3.5 ± 0.22 (39)	4.3 ± 0.06 (+7)	33
Putrescine	3.0 ± 0.06 (42)	2.8 ± 0.04 (30)	42
Spermidine	1.2 ± 0.02 (77)	1.0 ± 0.03 (62)	10
Spermine[a]	0.6 ± 0.02 (81)	0.8 ± 0.06 (64)	5

[a] Peeled leaves floated on spermine for 1 h; leaves then washed and floated on buffer for the remaining 47 h

Protease activity and chlorophyll loss were measured in leaves after 48 h incubation in the dark. Figures in parentheses represent % inhibition relative to final control

commonly reported proteases having pH optima at 4.2 and 6.6 with Azocoll as substrate. The acid protease increased >6-times the initial value during 48 h dark incubation, while the neutral protease activity increased only ~2.5-times. This rise in both protease activities was inhibited when leaves were floated on polyamine solutions. Spermine was most effective, followed by spermidine, putrescine and cadaverine. In all cases, the inhibition was greater for acid than for neutral proteases, and remained unaltered when the enzyme was either passed through Sephadex G-50 or exhaustively dialyzed against buffer in the cold and subsequently treated with the polyamine. These results indicate that protease activity is directly inhibited by polyamines and not mediated by a low M_r factor.

Determinations of chlorophyll content, a measure of leaf senescence, showed that during dark incubation for 48 h, the chlorophyll content in control leaves decreased by ~73% when compared with the initial value (table 1). Exogenous application of the polyamines prevented this decrease. As in the reduction of protease activity, the tetramine spermine and triamine spermidine were more effective than the diamines in preventing chlorophyll breakdown.

3.2. *In vitro inhibition of protease activity*

Various concentrations of polyamines were added to the enzyme extract during assay of enzyme activity. The results that both acid and neutral proteases are inhibited (fig.1). As before, the polyamines were more effective than the diamines. At lower concentrations of spermine and spermidine, the inhibition was more pronounced for acid than for neutral proteases. In contrast, cadaverine and putrescine produced only small decreases in both types of protease. With all polyamines, the inhibition of protease increased with increasing concentrations.

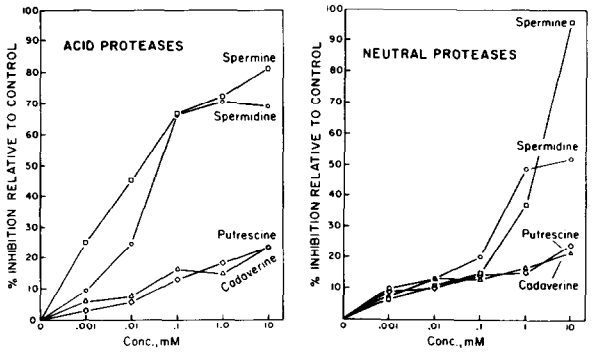

Fig.1. In vitro inhibition of protease activity in oat leaf extracts. Various concentrations of the polyamines were added to the enzyme extract at time of assay.

3.3. *Interaction of polyamines with enzyme or substrate*

To determine whether polyamines inhibit protease activity by interacting with enzyme or with substrate, spermidine (5 mM) was preincubated with enzyme or substrate preparations for 30 min at room temperature. The enzyme–polyamine mixture was then passed through Sephadex G-50 column or exhaustively dialysed, while the substrate–polyamine mixture was thoroughly washed prior to assay. The results in table 2 show that pre-treatment of the enzyme with spermidine prior to addition of substrate inhibited both the acid and neutral protease activities, while pre-treatment of the substrate with spermidine did not. This suggests that polyamines inhibit protease activity by binding to the enzyme.

3.4. *Effect of calcium on polyamine inhibition of protease activity and chlorophyll loss*

At physiological pH, the polyamines function as polyvalent cations and are known to bind strongly

Table 2

Interaction of spermidine with enzyme or substrate

	Protease activity (% inhibition)	
	Acid	Neutral
Enzyme	0	0
+ 1 mM spermidine pre-treated with 5 mM spermidine for 30 min at 24°C and passed through Sephadex G-50 column[a]	57 53	48 45
Substrate (Azocoll) pretreated with 5 mM spermidine and washed 4× prior to assay	0	0

[a] Final concentration of spermidine during assay was 1 mM

to the acidic groups of nucleic acids and proteins [18,19]. Thus, it is possible that polyamines may prevent senescence of leaves by virtue of their polyvalent cationic properties. If this were true, then treatments with other cations, such as Ca^{2+}, would be expected to reduce the effects of polyamines on inhibition of protease activity and chlorophyll breakdown. Exogenous application of $CaCl_2$ (1–10 mM) along with spermidine (1 mM) antagonized the spermidine-mediated inhibition of rise in acid protease activity and chlorophyll degradation. Treatments with Ca^{2+} alone reduced protease activity only slightly and did not prevent chlorophyll loss. In vitro application of Ca^{2+} produced no inhibition of protease activity (not shown).

4. DISCUSSION

The observed increases in protease activity followed by chlorophyll breakdown in excised oat leaves incubated in the dark, and their partial suppression by senescence-inhibiting polyamines indicate that protease, among other hydrolases, may be involved in leaf senescence. Analysis of extracts of oat leaves have revealed that they contain at least 2 proteases, most active at pH 4.2 and pH 6.6, which digest Azocoll, supporting [15,20]. The greater inhibition by polyamines of the rise in acid protease activity concurs with effects produced by kinetin and cycloheximide [15,20]. Thus the acid protease appears to be the functional protease during early senescence.

Although the mechanism by which polyamines inhibit protease activity is not clear at present, the differential extent of inhibition by the diamines and polyamines can probably be attributed to the number of free amino groups, which affect their cationic properties [1,2]. Thus, spermine, a tetraamine, is the most effective inhibitor, followed by the triamine spermidine, which in turn is more effective than the diamines putrescine and cadaverine. Similar results have been obtained on inhibition by polyamines of RNase activity in plants [9,21,22] and animal cells [23] and are further confirmed by the polyamine-mediated inhibition of chlorophyll loss in dark-incubated leaf segments of several leaves [8].

The inhibition of protease activity is not dependent on interaction with a low-M_r substance, because dialysed or Sephadexed enzyme extracts showed no loss of activity and no change in the extent of inhibition by spermidine when added in vitro. Spermidine thus appears to inhibit protease activity directly, perhaps by binding to the enzyme, as has been reported for certain RNases [22,23].

Since polyamines are known to act as cations at physiological pH [1,2] their action may involve electrovalent linkages with negatively charged groups of the enzyme or substrate. Such a linkage has been observed in several animal and bacterial systems [23], and in the inhibition of RNase activity in oat leaf protoplasts [22]. Our experiments support the view that spermidine inhibits protease activity by interacting with the enzyme and not the substrate, since decreased activity was observed only when spermidine was preincubated with the enzyme extract prior to the assay. The binding of spermidine to the crude enzyme from oat leaves is insensitive to EDTA, which suggests that binding is not the transglutaminase type as reported in animal cells [19]. Furthermore, since Ca^{2+} prevents the spermidine effect only when added in vivo, the protease inhibition may be mediated by an initial ionic attachment mechanism, in which Ca^{2+} and spermidine compete for the same ionic sites on membranes. A similar mechanism has been suggested previously in experiments on chlorophyll retention [8] and ethylene inhibition in oat leaves [24] and apple fruit tissue [25].

Many effects of polyamines may result from repression of de novo protease synthesis as well as by direct inhibition of protease activity. The rise in protease activity during dark senescence of leaves is inhibited by cycloheximide [20], suggesting its de novo synthesis. Consequently, the repression of protease activity by polyamines in vivo may be due to a specific inhibition of hydrolase synthesis. The direct inhibition of protease activity as manifested by the in vitro experiments probably results from neutralization of the negative sites on the enzyme by the positively charged polyamines thereby rendering it less accessible to the substrate. A similar mechanism has been proposed for RNases from oat leaf protoplasts [22], and bacterial and animal cells [23]. Polyamines are also known to stabilize plant membranes [26,27] and acid hydrolases from wheat [28] and from a number of plants [29] may be localized in the central vacuole, analogous to

that of the animal lysosome. Polyamines may therefore also act by restricting the release of proteases from the central vacuole.

Overall, results suggest a regulatory interaction between polyamines and the de novo synthesis and activity of proteases. The inhibition of protease activity in vitro by polyamines appears to be specific for oat leaves since similar in vitro experiments with papain (papaya latex, Sigma), bromelin (pineapple stem, Sigma) and trypsin (bovine pancreas, Sigma) showed no inhibition by spermidine over pH 4.2–8. Although the polyamines have been shown to interact with several enzymes concerned with the synthesis and breakdown of macromolecules, this is the first report on inhibition of protease activity by polyamines.

Thus, polyamines appear to prevent chlorophyll loss in excised oat leaves incubated in darkness by preventing an early senescence-linked rise in protease activity. This is presumably accomplished through electrovalent attachment of protonated amino groups to anionic sites on proteins. A similar action in plants also prevents a rise in and inhibition of RNase activity [9,21,22], stabilizes membranes to lysis [6], and leakage [26]. Decrease in polyamine biosynthesis with increased senescence in attached and excised oat leaves [10] further establishes the role of polyamines in leaf senescence.

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