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SENESCENCE OF RICE LEAVES

XI. Inhibition of Protease Activity by α, α'-Dipyridyl

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Abstract

The effect of α, α'-dipyridyl (DP) on protease activity in connection with its action in controlling chlorophyll loss of detached rice leaves was investigated. The first detactable DP effect was the retardation of the increase in free amino nitrogen and decrease in protein, which occurred at 2 days after incubation. However, retardation of chlorophyll loss by DP was observed 3 days or more after the initiation of the experiments, but not before. The protease activity was inhibited when detached leaves were floated on DP solution. *In vitro* protease activity was also inhibited by DP. The possible mechanism of DP on protease activity was discussed.

Introduction

One of the early events in leaf senescence is the well-documented rise in protease activity (Thimann, 1980). However, little is known about the regulation of proteolysis itself during leaf senescence. Since exogeneous application of α, α'-dipyridyl (DP) or other metal chelators retarded dark senescence of detached rice leaves, we postulated that the effect of chelator may be related to the inhibition of protease activity (Cheng and Kao, 1984). We therefore investigated the effects of DP on protease activity in connection with its action in controlling senescence of detached rice leaves.

Materials and Methods

Plant Material and Incubation Condition

Rice (*Oryza sativa* L. cv. Taichung Native 1) seedlings were cultured as described elsewhere (Kao, 1980b). The apical 3 cm of the third leaves of 10-day-old

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seedlings was used for experiments. Ten rice-leaf segments were floated on 10 ml of water or DP solution in a 50-ml flask. The flasks were then incubated at 27°C in darkness.

Determinations of Chlorophyll, α-amino Nitrogen and Protein

Chlorophyll, α-amino nitrogen and protein were extracted and determined as described before (Kao, 1980a, b), and expressed as A_{665}, A_{570} and A_{700} per ten segments, respectively.

Enzyme Extraction

Leaf segments weighing about 200 mg were homogenized in a pre-chilled mortar and a pestle with 10-ml of cold 50 mM tris-meleate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol at 4°C. The homogenates were centrifuged at 20,000 g for 20 min at 4°C. The resulted clear supernatant fractions were assayed for protease activity.

Enzyme Assay

Protease activity was assayed using acid-denatured hemoglobin (Sigma Chem. Co.) as substrate, since it is readily soluble at fairly high concentrations over a wide range of pH. Preliminary experiments showed that protease had optimum activity at pH 4.5. Therefore, protease activity was assayed at acid condition. The reaction mixture for the acid protease consisted of 1.5 ml sodium acetate buffer (0.05 M, pH 4.5) containing 0.1% 2-mercaptoethanol, 0.5 ml of 2% hemoglobin (dissolved in water), and 1.0 ml of enzyme solution. Reactions were carried out at 40°C on a shaking water bath for 90 min, and were terminated by the addition of 1 ml of 20% trichloroacetic acid. Blanks were prepared by adding trichloroacetic acid prior to the addition of enzyme. Insoluble protein was removed by centrifugation, and α-amino nitrogen in the supernatant was determined as described before (Kao, 1980a). Enzyme activity was expressed as μmoles of α-amino nitrogen liberated per g fresh weight per hour. Specific activity was expressed as μmoles of α-amino nitrogen liberated per mg protein per hour.

Results and Discussion

The results in Figs. 1 and 2 generally confirmed our earlier work that during rice leaf senescence proteolysis preceded the degradation of chlorophyll (Kao, 1978). These results also demostrated that the first detectable DP effect was the retardation of the increase in free amino nitrogen and the decrease in protein, which occurred at 2 days after incubation (Figs. 1B and 2). On the other hand, retardation of chlorophyll loss by DP was observed 3 days after the initiation of the experiments, but not before.

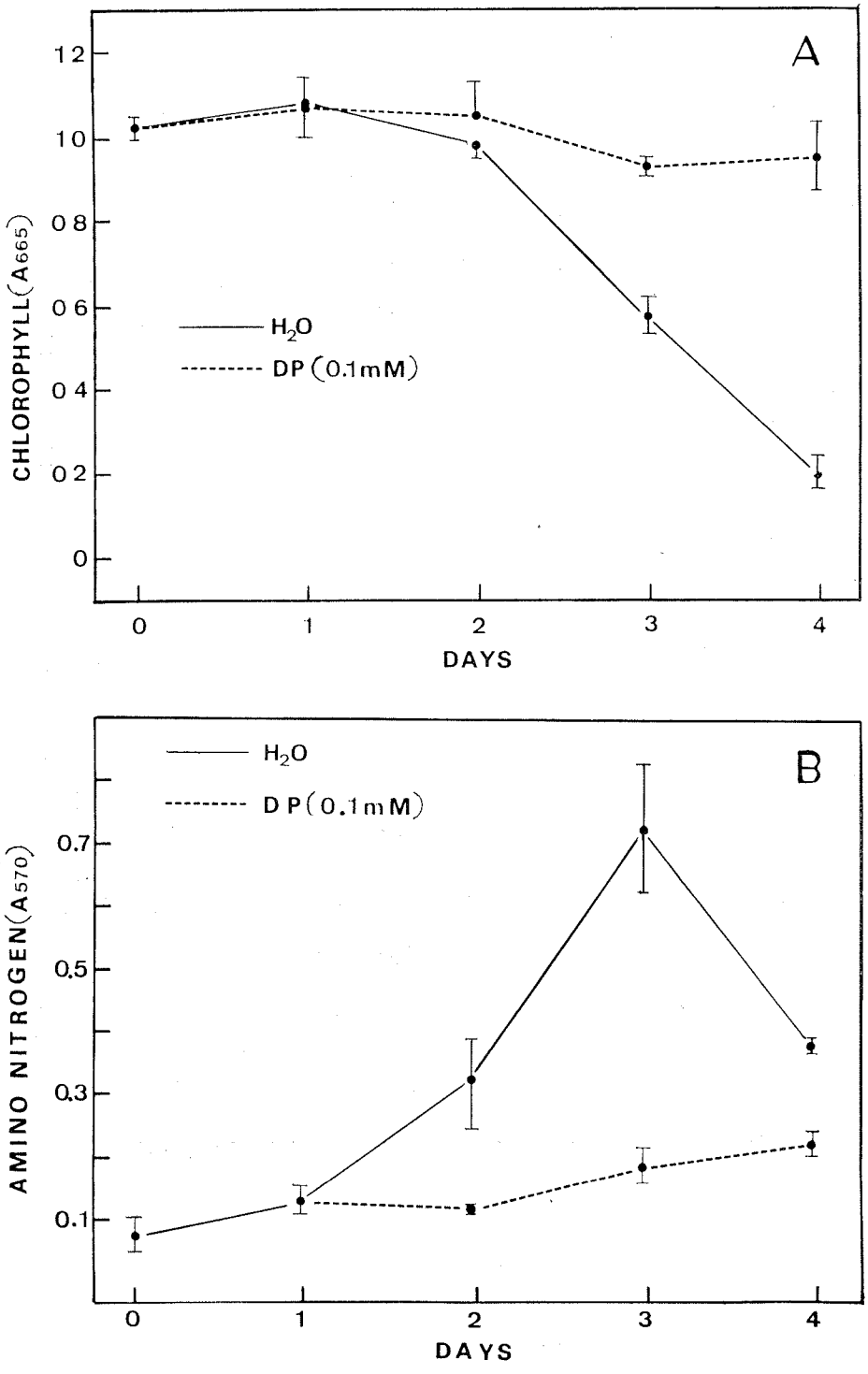

Fig. 1. Time courses of change of chlorophyll (A) and amino nitrogen (B) levels of detached rice leaves incubated in water and DP under dark condition.

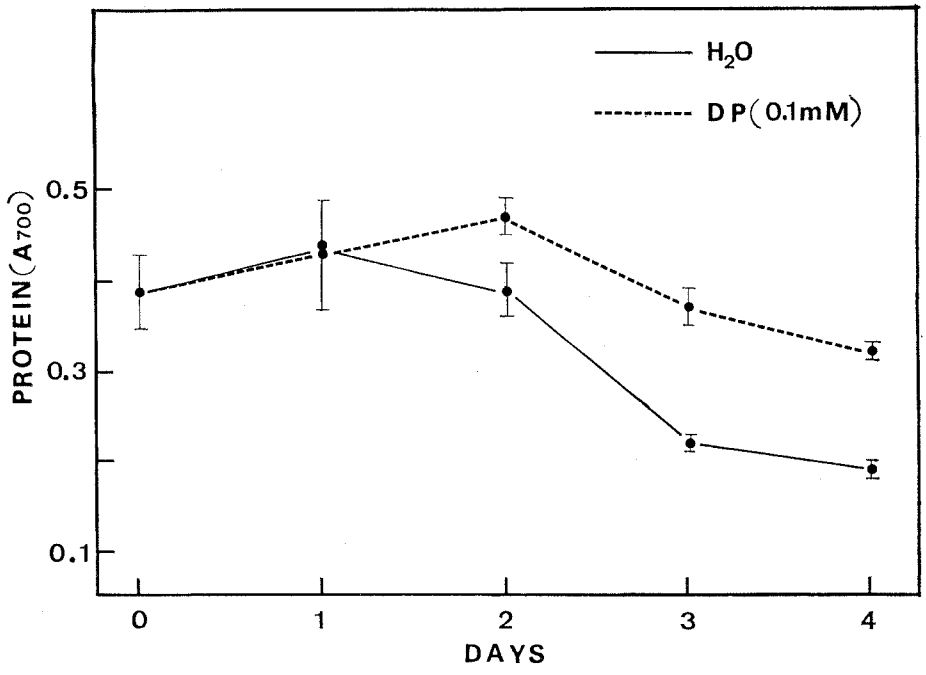

Fig. 2. Time courses of change of protein level of detached rice leaves incubated in water and DP under dark condition.

It has been proposed that the process of senescence is a sequential one in which the formation of proteolytic enzyme is of prime importance (Kao, 1978; Martin and Thimann, 1972). Therefore, one possible interpretation of the above results is that DP retards chlorophyll degradation through the inhibition of proteolysis, either the formation of protease or the activity of protease. If so, the *in vivo* or *in vitro* protease activity should be inhibited by DP.

Our unpublished data indicated that the rapid decline of protein in detached rice leaves during senescence was associated with the increase of acid protease activity using hemoglobin as substrate. This increase in protease activity was inhibited when leaf segments were floated on DP solution (Table 1). *In vitro*

Table 1. ***In vivo*** *inhibition of protease activity by 0.1 mM DP*

Protease activity in rice leaf segments were assayed after 4 days of incubation in darkness. The figures given in the parentheses represent the percentage inhibition of control.

Treatment	Activity (μ moles NH_2 g^1 h^{-1})	Specific activity (μ moles NH_2 mg^{-1} protein h^{-1})
Control	48.9±2.0	4.4
DP	1.4±1.2 (56)	1.0 (68)

inhibition of protease activity by DP or other metal chelators were determined by incubating DP or other metal chelators with enzyme solution prior to standard protease assay. Results in Fig. 3 show that DP or other chelators strongly inhibited *in vitro* protease activity. Since enzyme activity was remained unaltered when the enzyme was exhaustively dialysed against buffer in the cold (data not shown), protease activity seems to be directly inhibited by DP and not mediated by a low molecule factor.

The rise in portease activity during dark senescence of oat leaves was inhibited by cycloheximide, a protein synthesis inhibitor (Martin and Thimann, 1972). Our unpublished work also demonstrated that protease activity was strongly inhibited by cycloheximide. Furthermore, DP has been shown to prevent the hydroxylation of proline (Barnett, 1970; Hurych and Chvapil, 1965). All of these results seems to suggest that protease is *de novo* synthesized during senescence. Therefore, the inhibition of protease activity by DP *in vivo* may be due to a specific inhibition of protease synthesis.

DP has been demonstrated to inhibit the activity of some metal-containing peptidases (Vallee and Neurath, 1955). The direct inhibition of protease activity as shown in the *in vitro* experiment (Fig. 3) probably resulted from chelation of

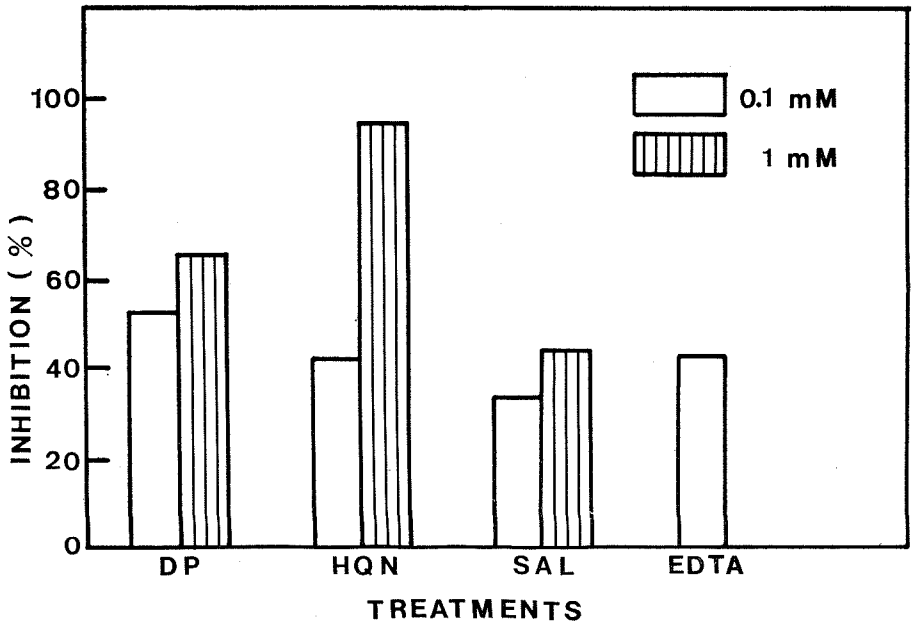

Fig. 3. *In vitro* inhibition of protease activity by DP and other metal chelators. The enzyme extract was incubated in metal chelator solutions for 30 min at 30°C prior to standard hemoglobin assay. HQN, SAL and EDTA are 8-hydroxyquinoline, salicylaldoxime and ethylenediaminetraacetic acid, respectively.

metal ion on the enzyme. It is not known from our observation what metal ion is being chelated. It is unlikely due to the chelation of Ni^{2+} and Co^{2+}, since Ni^{2+} and Co^{2+} clearly retard dark senescence of rice leaf segments (Kao and Yu, 1981). The inhibition of protease activity *in vitro* by DP appears to be specific for rice leaves since similar *in vitro* experiments with oat leaves showed no inhibition by DP (Drivdahl and Thimann, 1978).

In conclusion, DP seems to prevent chlorophyll loss in detached rice leaves incubated in darkness by preventing an early senescence-linked increase in protease activity.

Acknowledgements

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水稻葉片老化之研究

(十一) α, α′-dipyridyl 與蛋白質水解酵素活性

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本研究主要探討 α, α′-dipyridyl (DP) 對水稻切離片蛋白質水解酵素活性之影響，以明瞭 DP 控制葉綠素分解之機制。在黑暗中 DP 處理水稻切離葉片二天即表現延緩蛋白質分解與氨基酸累積之效應，而 DP 必須處理三天後才能表現延緩葉綠素之分解。切離葉片以 DP 處理後，蛋白質水解酵素活性被抑制；此外，蛋白質水解酵素直接以 DP 處理亦抑制酵素活性。DP 抑制蛋白質水解酵素活性之機制在本文中有詳細之討論。DP 很可能是抑制蛋白質水解酵素活性而延緩葉綠素之分解。