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*Short Communication*

# Synthesis and Degradation of Nitrite Reductase in Pea Leaves[1]

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## ABSTRACT

**We have shown in a previous publication (Gupta, Beevers 1983 J Exp Bot 34: 1455-1462) that the level of extractable nitrite reductase activity in pea (*Pisum sativum* cv Burpeeana) leaves is subject to environmental perturbations. In the current study, we have used rocket immunoelectrophoresis to quantitate the level of nitrite reductase protein in extracts from pea plants subjected to various environmental treatments. The level of nitrite reductase cross-reacting material closely followed the level of assayable nitrite reductase activity. The environmental conditions which enhanced the level of extractable nitrite reductase activity resulted in an increased level of nitrite reductase cross-reacting material in the extracts. In contrast, environmental conditions which resulted in a decrease in the level of extractable nitrite reductase activity produced a decline in cross-reacting material. These results indicate that the environmentally induced modulation of extractable nitrite reductase activity involves alteration of enzyme level and is not mediated by a reversible activation-inactivation of the existing enzyme.**

Nitrite reductase is the second enzyme in the pathway of nitrate reduction. In similarity to nitrate reductase, the first enzyme in the pathway, the level of extractable nitrite reductase activity is influenced by environmental fluctuations.

Various mechanisms have been described to account for the environmentally modulated fluctuations in assayable nitrate reductase activity. In *Chlorella*, the photoregulation of extractable nitrate reductase appears to involve an activation-inactivation mechanism (7). By using immunological methods, Funkhouser and Ramadoss (1) concluded that the increase in extractable nitrate reductase activity when ammonium-grown *Chlorella* is transferred to nitrate is also due to activation of a prexisting enzyme protein. In contrast to these findings, Somers *et al.* (6) concluded that the modulation of extractable nitrate reductase level in barley by light and nitrate involves *de novo* synthesis and degradation of the enzyme and is not accounted for by an activation-inactivation mechanism.

No previous studies have speculated on the mechanism of environmental modulation of nitrite reductase level. We have used rocket immunoelectrophoresis to quantitate the level of nitrite reductase in extracts from pea leaves subjected to different environmental treatments. We conclude that fluctuations in the level of extractable nitrite reductase are modulated by an alteration of enzyme level and are not due to an activation-inactivation mechanism.

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## MATERIALS AND METHODS

Pea (*Pisum sativum* L. cv Burpeeana; Burpee Seed Co., Warminster, PA) seeds were germinated and environmental variations were imposed as described previously (2). At predetermined time intervals, 1.0 g fresh weight of leaves were taken from three plants picked at random and frozen until all of the samples had been collected.

Crude homogenates prepared according to Gupta and Beevers (2) in extraction buffer consisting of 100 mM K-phosphate (pH 8.8), 5 mM EDTA, and 1 mM cysteine-HCl were fractionated with saturated $(NH_4)_2SO_4$. The material precipitated between 40 and 75% saturation of $(NH_4)_2SO_4$ was pelleted by centrifugation at 10,000*g* for 25 min and resuspended in 0.5 ml of 50 mM K-phosphate (pH 7.5) containing 10% (v/v) glycerol. The resuspended pellets were desalted on small Sephadex G-25 columns (1.5 × 5.0 cm) under centrifugation. This desalted enzyme extract was used for rocket immunoelectrophoresis.

Preparation of antiserum to Fd-affinity purified nitrite reductase and rocket immunoelectrophoresis were performed as described earlier (3).

Nitrite reductase activity in extracts was assayed by the method of Ida and Morita (4). One unit of enzyme activity is defined as the reduction of 1 $\mu$mol $NO_2^-h^{-1}$. Nitrite reductase was localized on the immunoelectrophoretic plates by a modification of the method of Lund and DeMoss (5). The gel was incubated with reduced methyl viologen and nitrite instead of nitrate. Protein on the immunoelectrophoresis plates was detected by Coomassie Blue stain (3).

## RESULTS AND DISCUSSION

Serial dilutions of partially purified pea nitrite reductase (extract after Sephacryl S-200 gel filtration) were run on agarose gel containing antiserum and, after the electrophoresis, the gel was stained for nitrite reductase activity. The rockets formed by the reaction of enzyme and antibodies are catalyticaly reactive (Fig. 1A). The gels were then stained for protein (Fig. 1B). The most distinct rockets in protein stained gel (Fig. 1B) corresponded in heights with rockets in enzyme stained gel (Fig. 1A). However, the distinct rockets in protein stained gel (Fig. 1B, well 2) were surrounded by another faint precipitin arc, apparently of non-specific origin. Serial dilution of the enzyme preparation resulted in a linear relationship between the amount of enzyme activity and the heights of the distinct rockets. With increasing enzyme activity, there was an increase in rocket height (Fig. 1, A and B).

When 13-d water-grown seedlings were kept on water or transferred to darkness and irrigated with 10 mM $KNO_3$ solution for 4 d, there was no increase in extractable activity above the basal level and we did not observe any increase in rocket height (Table I), *i.e.* there was little or no nitrite reductase cross-reacting material present in the extracts of the above seedlings. However, when pea seedlings receiving nitrate in the nutrient solution were

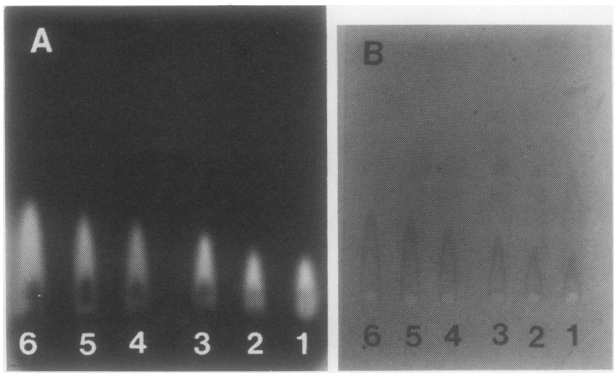


FIG. 1. Rocket immunoelectrophoresis of serially diluted partially purified nitrite reductase from pea leaves on 0.9% (w/v) Sea plaque agarose plate containing 0.4% (v/v) antiserum. Wells contained (1) 0.18 unit, (2) 0.36 unit, (3) 0.54 unit, (4) 0.72 unit, (5) 0.9 unit, and (6) 1.1 units of nitrite reductase activity. Plates were electrophoresed at 50 v and 5 mamps for 3 h at 4°C. After the electrophoresis, the wells were filled with 1% (w/v) agarose and stained for nitrite reductase activity (A), the nitrite reductase/anti-nitrite reductase complex appeared as colorless rockets in the blue background. After washing the plate with 1% (w/v) NaCl and water, the gel was dried and stained with Coomassie blue for protein staining (B).

Table I. *Effect of Environmental Variations on Nitrite Reductase Activity and Nitrite Reductase Cross-Reacting Material in Pea Leaves*

Water-grown pea seedlings were subjected to nitrate, light, shade and stress conditions. Nitrite reductase activity was measured and an aliquot of the extacts was used to estimate nitrite reductase cross-reacting material by rocket immunoelectrophresis.

| Treatment | NiR[a] Activity | Nir CRM[b] Rocket Height |
|---|---|---|
| | *μmol $h^{-1}$ $g^{-1}$ fresh wt* | *mm* |
| Control, zero time | 2.5 | 3.0 |
| 4 d light, water | 2.6 | 3.0 |
| 4 d dark, nitrate | 2.8 | 2.0 |
| 6 d light, nitrate | 18.8 | 11.1 |
| 2 d light, nitrate + 5 d dark, nitrate | 14.2 | 9.4 |
| 2 d light, nitrate + 5 d light, water | 8.4 | 7.9 |
| 2 d light, nitrate + 5 d light, stress | 7.1 | 7.5 |

[a] Nitrite reductase.
[b] Nitrite reductase cross-reacting material.

illuminated, there was a gradual increase in enzyme activity as well as an increase in nitrite reductase cross-reacting material as indicated by an increase in the rocket heights detected by protein staining or enzyme assay. Maximal enzyme activity was attained on day 6 of nitrate and light-treated seedlings and extracts showed a maximal rocket height of 11.1 mm (Table I). Thus, the nitrate- and light-mediated increase in enzyme activity is associated with a corresponding rise in the amount of immunoprecipitable material.

When nitrate was withheld from 2-d induced plants, following an increase in activity after the 1st d (2), nitrite reductase activity declined and there was corresponding increase and decrease in the rocket heights (Fig. 2) which reflected the amount of nitrite reductase cross-reacting material present in the extracts. At day 5 (Fig. 2, well 6), the rocket height was minimum (Table I) which corresponded to the lowest nitrite reductase activity in the extract.

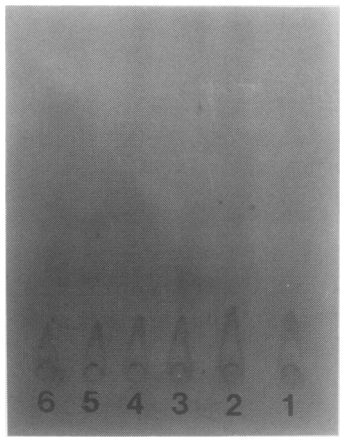


FIG. 2. Rocket immunoelectrophoresis of enzyme extracts of leaves taken from pea seedlings transferred to nitrate-depleted medium, six wells were punched on agarose gel containing antiserum and filled with: (1) extract at zero day of transfer, 0.17 unit; (2) 1st d of transfer, 0.20 unit; (3) 2nd d of transfer, 0.17 unit; (4) 3rd d of transfer, 0.15 unit; (5) 4th d of transfer, 0.14 unit; and (6) 5th d of transfer, 0.10 unit. The gel was subjected to electrophoresis and stained for protein as described above.

Similarly, when induced plants were transferred to noninducing conditions such as darkness and water stress for 5 d, the decrease in assayable activity of nitrite reductase was reflected in a decline in nitrite reductase cross-reacting material (Table I) as indicated by a decrease in rocket height.

We have shown that nitrate and light are both required to induce the nitrite reductase activity in the pea leaf (2) and the induction of nitrite reductase activity is associated with an increase in nitrite reductase cross-reacting material, indicating that there is synthesis of new enzyme protein in response to light and nitrate. Very little nitrite reductase cross-reacting material was detectable in the extracts from uninduced plants.

The decline in nitrite reductase extractable activity when the induced plants are subjected to noninducing conditions seems to be modulated by the disappearance of enzyme protein or degradation of protein into nonimmunologically reactive form. It could be argued that by $(NH_4)_2SO_4$ precipitation we were selecting out only native forms of the enzyme. However, in our unpublished data we have shown that we immunoprecipitate only one polypeptide corresponding to nitrite reductase subunit from crude extracts prepared from induced plants. Assuming that the antibodies would recognize any inactive enzyme, an activation-inactivation mechanism is precluded.

The above situation is very similar to the one reported for barley nitrate reductase (6). Thus, it is reasonable to conclude that modulation of nitrite reductase activity in response to environmental perturbations appears to be due to *de novo* synthesis and degradation and not to reversible activation-inactivation of the enzyme protein. This is the first report on the regulatory mechanisms of nitrite reductase activity. Work is in progress to determine whether this control of enzyme synthesis is exerted at the transcriptional or translational level.

## LITERATURE CITED

1. FUNKHOUSER EA, CS RAMADOSS 1980 Synthesis of nitrate reductase in *Chlorella*. II. Evidence for synthesis in ammonia-grown cells. Plant Physiol 65: 944–948
2. GUPTA SC, L BEEVERS 1983 Environmental influences on nitrite reductase activity in *Pisum sativum* L. seedlings. J Exp Bot 34: 1455–1462
3. GUPTA SC, J FLETCHER, L BEEVERS 1983 Purification and charactization of nitrite reductase from cell suspension cultures of Paul's Scarlet Rose and its cross reactivity to antiserum prepared against pea leaf nitrite reductase. Z Pflanzenphysiol. In press
4. IDA S, Y MORITA 1973 Purification and general properties of spinach leaf nitrite reductase. Plant Cell Physiol 14: 661–671
5. LUND K, JA DEMOSS 1976 Association-dissociation behaviour and sub-unit structure of heat-released nitrate reductase from *Escherichia coli*. J Biol Chem 251: 2207–2216
6. SOMERS DA, TM KUO, A KLEINHOFS, RL WARNER, A OAKS 1983 Synthesis and degradation of barley nitrate reductase. Plant Physiol 72: 949–952
7. TISCHNER R, A HUTTERMANN 1978 Light-mediated activation of nitrate reductase in synchronous *Chlorella*. Plant Physiol 62: 284–286