The intracellular location of nitrogen metabolism enzymes in *Pisum arvense* L. roots

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Abstract

The time course changes of nitrate assimilation enzymes and their distribution has been studied in *Pisum arvense* roots. The results indicate that nitrate reductase (EC 1.6.6.2) and glutamine synthetase (EC 6.3.1.2) are present in the soluble fraction, and nitrite reductase (EC 1.6.6.4) and glutamate synthase (EC 2.6.1.53) are localised in the plastids. The results show that the glutamine synthetase/glutamate synthase system is the major pathway of ammonium incorporation in NO_3^--supplied *Pisum arvense* roots and glutamate dehydrogenase plays a lesser role.

Key words: nitrogen, assimilation enzymes — intracellular location.

INTRODUCTION

Nitrate reductase and nitrite reductase are the first enzymes which participate in the conversion of NO_3^- to NO_2^- and NH_4^+ (for review see Beevers and Hageman 1969, Hewitt 1975, Murphy et al. 1974). The ammonium ions are further incorporated into the amino acids by glutamate dehydrogenase or glutamine synthetase/glutamate synthase (Miflin and Lea 1977).

Most studies on subcellular localisation of enzymes involved in nitrate assimilation were performed with leaf tissues of many plant species. Dalling et al. (1972), Miflin (1974), Emes and Fowler (1979) and Suzuki et al. (1981) examined the localisation of nitrate assimilation enzymes in the roots of *Triticum aestivum, Zea mays, Oriza sativa, Pisum sativum* and *Hordeum vulgare*. These authors suggested that nitrite reductase and glutamate synthase are localised in plastids, while nitrate reductase and glutamine synthetase are present in the cytosol.

MATERIAL AND METHODS

Seeds of the field pea (*Pisum arvense* L., cv. Nieznaniecka) were surface-sterilized in 2 per cent calcium hypochlorite. After rinsing the seeds were germinated in distilled water in darkness at 27°C for 3 days. Uniform seedlings were transplanted onto perforated aluminium foil stretched over a 800 cm^3 beaker filled with precultured solution containing (mmoles×dm^{-3}): K_2SO_4 3.0, $Ca(H_2PO_4)_2$×$2H_2O$ 1.0, $CaSO_4$×$2H_2O$ 2.0, $MgSO_4$×$7H_2O$ 1.0, ferric citrate 0.017 and micronutrients (μmoles×dm^{-3}): H_3BO_3 3.0, $MnSO_4$×$5H_2O$ 1.0, $CuSO_4$×$5H_2O$ 1.0, $ZnSO_4$×$7H_2O$ 0.1, $CoSO_4$×$7H_2O$ 0.01 and Na_2MoO_4 0.001. pH was adjusted to 6.5. After 3 days growth of seedlings on this medium in a growth chamber under a light-darkness photoperiod (17 h light, 5000 lux) at 25°C and 48 per cent relative humidity, the plants were transferred to Hoagland nitrate solution with microelements in the same light–darkness conditions as described above.

The fresh roots were suspended in grinding medium and ground with a mortar and pestle. The resulting homogenate was filtered through 4 layers of cheesecloth and subsequently centrifuged at 16000×g for 10 min at 2°C. The supernatant was used for enzyme assay.

Extracts of nitrate reductase (NR) and nitrite reductase (NiR) were prepared according to the method described previously (B u c z e k 1976). The method of H i p k i n and S y r e t t (1977) was used for extraction of glutamine synthetase (GS). Glutamate synthase (GOGAT) was prepared by the method described by E m e s and F o w l e r (1979).

The particular crude enzyme extracts for differential centrifugation were obtained according to the above described procedure. Each grinding medium contained also 0.2 M sucrose and 0.3 M mannitol. Differential centrifugation was conducted according to M i f l i n (1970) except that the first centrifugation was run at 600×g for 5 min. The pellets obtained in each step were washed with the same buffers, recentrifuged and finally suspensed in small amounts of appropriate grinding media without sucrose and mannitol but with addition of 0.1 per cent Triton X-100. After standing for 30 min pellets were recentrifuged and the supernatants were used for enzyme assay.

NR activity was assayed according to the method of H a g e m a n and F l e s h e r (1960) by measuring NADH-dependent production of NO_2^-. NiR activity was measured by following the disappearance of nitrite with the use of dithionite-reduced methylviologen as reductant (H u c k - l e s b y et al. 1972). The method of H i p k i n and S y r e t t (1977) was used for GS activity assay. The absorbance of the γ-glutamylhydroxamate was read at 500 nm. GOGAT activity was assayed by the method described by E m e s and F o w l e r (1979). Glutamate dehydrogenase (GDH) activity was assayed by the method of D u k e et al. (1975), glucose-6-

-phosphate dehydrogenase according to D e v l i n and G a l l o w a y (1968) and cytochrome c oxidase as described by S m i t h (1955). Soluble protein was determined according to L o w r y et al. (1951).

The total activity of GDH and GS is expressed in μmoles and GOGAT, NR and NiR in nmoles of product formed or substrate used per gram of fresh weight per minute. The specific activity of these enzymes was expressed in μmoles or nmoles of product or substrate per mg of protein per minute.

Each value in the tabeles and figures represents a mean of three replications and each experiment was repeated at least three times.

<div align="center">RESULTS</div>

The time-course changes of nitrate assimilation enzymes in field pea roots are shown in Fig. 1. The activity of NiR, GS and GOGAT rose from the 1st to the 6th day of growth of young plants in nitrate-containing nutrient solution. These enzymes attained their plateau either on the fifth or sixth day after transferring the plants to the nitrate solution. On the other hand, NR activity increased rapidly and attained its maximum on the third day, however, thereafter, the enzyme activity decreased and remained constant for six days. It seems that GDH plays a lesser role in NO_3^- assimilation, because its activity was very low and after the initial increase on the first day of growth of plants in nitrate solution, its activity decreased with time.

Table 1 gives the distribution of nitrate assimilation enzymes obtained from *Pisum arvense* roots after six days growth of plants ni nitrate--containing solution. The results show that NR and GS are associated almost exclusively with the soluble (cytosol) fraction. On the other hand, the activity of NiR and GOGAT was relatively high in the soluble fraction but in contrast to nitrate reductase and glutamine synthetase some portions of NiR and GOGAT activities were associated with the organelle fractions. It seems on the basis of marker enzymes (for mitochondria — cytochrome c oxidase and for plastids — glucose-6-phosphate dehydrogenase) presented in Table 2 that NiR and GOGAT are associated with the fraction of plastids.

<div align="center">DISCUSSION</div>

Preliminary studies on the changes of nitrate assimilation enzymes in field pea roots during 14 days growth of plants showed some variations in their activities. The pronounced decrease of GDH activity suggests that this enzyme plays a minor role in the assimilation of NO_3^- absorbed
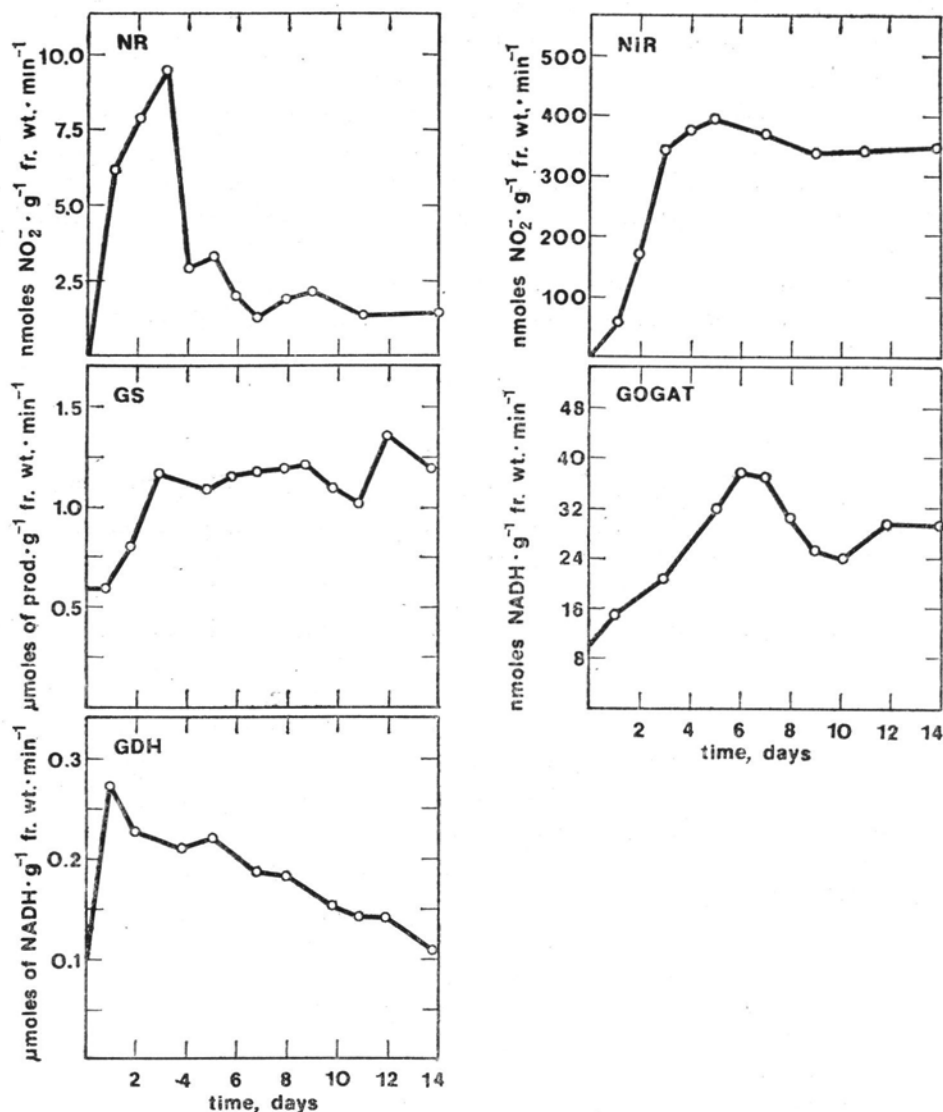
Fig. 1. Time-course changes of nitrate assimilation enzymes during 14-day growth of plants. NR — nitrate reductase; NiR — nitrite reductase; GS — glutamine synthetase; GOGAT — glutamate synthase; GDH — glutamate dehydrogenase

by *Pisum arvense* roots. This interpretation of our data is consistent with Miflin and Lea's suggestion for higher plant tissues presented in their paper (1977). It is well known that GDH having a relatively high K_m towards NH_4^+ (Lea and Thurman 1972) can probably assimilate ammonia only when intracellular NH_4^+ concentration is especially high (Shepard and Thurman 1973). It seems that when NO_3^- is the source of nitrogen, the GS/GOGAT system should be operating as the

Table 1

Activity of nitrate assimilation enzymes and their distribution between the pellet and supernatant
fractions obtained from field pea roots

Fraction	NR		NiR		GS		GOGAT	
	activity[1]	%	activity[1]	%	activity[2]	%	activity[3]	%
Filtra$_{te}$	0.54	100	17.4	100	0.27	100	4.6	100
Supernatant: 600×g	0.53	98	19.7	113	0.31	120	3.6	90
4700×g	0.61	113	21.9	126	0.35	136	3.1	77
8400×g	0.48	89	25.0	144	0.39	151	3.4	85
21500×g	0.59	109	28.1	161	0.37	146	2.9	72
Pellet: 600×g	0.01	2	4.5	26	0.03	12	7.0	1175
4700×g	0.11	18	22.2	127	0.017	6	14.2	355
8400×g	0.04	7	9.7	56	0.0	—	1.8	45
21500×g	0.08	15	4.5	26	0.095	37	1.0	25

Specific activity of enzymes: [1] — nmoles NO_2^- mg^{-1} protein min^{-1}; [2] — μmoles of product mg^{-1} protein min^{-1}; [3] — nmoles NADH mg^{-1} protein min^{-1}.

Table 2

Activity of cytochrome c oxidase and glucose-6-phosphate dehydrogenase in pellets obtained during differential centrifugation of field pea roots

Fraction	Cytochrome c oxidase, nmoles of substrate $\times g^{-1}$ fr.wt. $\times$ min^{-1}	Glucose-6-phosphate dehydrogenase, nmoles NADPH $\times g^{-1}$ fr.wt. $\times$ min^{-1}
$600 \times g$	3.5	48.2
$4700 \times g$	5.2	385.8
$8400 \times g$	16.8	96.4
$21500 \times g$	7.7	144.6

major assimilatory pathway in *Pisum arvense* roots. M i f l i n and L e a (1977) and F o w l e r and B a r k e r (1978) have advanced the hypothesis that ammonia incorporation in many non-photosynthetic higher plant tissues occurs through the glutamine synthetase/glutamate synthase pathway.

The slight nitrate reductase activity associated with pellet fractions is distinct evidence for the localisation of this enzyme in cytosol. Similar experimental support of this view has been obtained by D a l l i n g et al. (1972), E m e s and F o w l e r (1979) and S u z u k i et al. (1981), however, the experiments described here are in contrast to the report by B u t z and J a c k s o n (1977). These authors suggested that NR may be linked with plasmalemma and/or chloroplast envelope membrane. It is very possible however, that the enzyme may by very loosely associated with these membranes and could be released during disruption of the cell.

In contrast to nitrate reductase the major activity of nitrite reductase within the pellet fraction concided with glucose-6-phosphate dehydrogenase activity indicating a plastid localisation of this enzyme. The level of recovery of nitrite reductase in the plastid fraction found in our experiment in *Pisum arvense* roots is similar to that obtained by D a l - l i n g et al. (1972) for wheat roots, but differs from that obtained by M i f l i n (1974) and E m e s and F o w l e r (1979) for *Pisum sativum*. It is worth noting that glucose-6-phosphate dehydrogenase is not a perfect marker for plastid identification, and some authors (E m e s and F o w l e r 1979, S u z u k i et al. 1981) used triose phosphate isomerase for plastid identification. It is a well known fact however, that this enzyme shows different isoforms both in cytosol and plastid fractions (H e r b e r t et al. 1979).

The major specific activity of glutamate synthase was also associated with the plastid fraction and there was no association with other cell organelles. Thus, the present work confirms the earlier data reported by E m e s and F o w l e r (1979) and S u z u k i et al. (1981) that glutamate synthase is possibly plastid-localised in non-photosynthetic cells of higher plants.

The data for glutamine synthetase estimated by the transferase reaction suggest that the enzyme is cytosol-localised. This is in agreement with the earlier observations of S u z u k i et al. (1981) conducted on roots of several plant species. However, E m e s and F o w l e r (1979) suggested that GS in *Pisum sativum* roots is connected partly with the cytosol and plastid fraction. Our results show however, that GS in *Pisum arvense* roots is cytosol-localised rather than associated with some organelle.

REFERENCES

B e e v e r s L., H a g e m a n R. H., 1969. Nitrate reduction in higher plants. Ann. Rev. Plant Physiol. 20: 495-522.

B u c z e k J., 1976. The role of light in the induction of nitrate reductase and nitrite reductase in cucumber seedlings. Acta Soc. Bot. Pol. 45: 77-92.

B u t z R. G., J a c k s o n W. A., 1977. A mechanism for nitrate transport and reduction. Phytochemistry 16: 409-417.

D a l l i n g M. J., T o l b e r t N. E., H a g e m a n R. H., 1972. Intracellular location of nitrate reductase and nitrite reductase. II. Wheat roots. Bichim. Biophys. Acta 283: 513-519.

D e v l i n R. M., G a l l o w a y R. A., 1968. Oxidative enzymes and pathways of hexose and triose metabolism in *Chlorella*. Physiol. Plant. 21: 11-25.

D u k e S. H., K o u k k a r i W. L., S o u l e n T. K., 1975. Glutamate dehydrogenase activity in roots: distribution in a seedling and storage root, and the effects of red and far-red illuminations. Physiol. Plant. 34: 8-13.

E m e s M. J., F o w l e r M. W., 1979. The intracellular location of the enzymes of nitrate assimilation in the apices of seedling pea roots. Planta 144: 249-253.

F o w l e r M. W., B a r k e r R. D. J., 1978. Assimilation of ammonium in non-chlorophyllous tissue. In: Nitrogen assimilation of plants. Hewitt E. J., Cutting C. V. (eds.), Academic Press, London, pp. 489-500.

H a g e m a n R. H., F l e s h e r D., 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 34: 700-708.

H e r b e r t M., B u r k h a r d Ch., S c h n a r r e n b e r g e r C., 1979. A survey for izoenzymes of glucosephosphate isomerase, phosphoglucomutase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in C_3-, C_4- and Crassulacean-Acid-Metabolism plants, and green alge. Planta 145: 95-104.

H e w i t t E. J., 1975. Assimilatory nitrate-nitrite reduction. Ann. Rev. Plant Physiol. 26: 73-100.

H i p k i n C. R., S y r e t t P. J., 1977. Some effects of nitrogen-starvation on nitrogen and carbohydrate metabolism in *Ankistrodesmus braunii*. Planta 133: 209-214.

H u c k l e s b y D. P., D a l l i n g M. J., H a g e m a n R. H., 1972. Some properties of two forms of nitrite reductase from con (*Zea mays* L.) scutellum. Planta 104: 220-233.

Lea P. J., Thurman D. A., 1972. Intracellular location and properties of plant L-glutamate dehydrogenases. J. Exp. Bot. 23: 440-449.

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Miflin B. J., 1970. Studies on the sub-cellular location of particulate nitrate and nitrite reductase, glutamic dehydrogenase and other enzymes in barley roots. Planta 93: 160-170.

Miflin B. J., 1974. The location of nitrite reductase and other enzymes related to amino acid biosynthesis in the plastids of root and leaves. Plant. Physiol. 54: 550-555.

Miflin B. J., Lea P. J., 1977. Amino acid metabolism. Ann. Rev. Plant Physiol. 28: 299-329.

Murphy M. J., Siegel L. M., Tove S. R., Kamin H., 1974. Siroheme: A new prosthetic group participating in six-electron reduction catalyzed by both sulphite and nitrite reductases. Proc. Nat. Acad. Sci. U.S.A. 71: 612-616.

Shepard D. V., Thurman D. A., 1973. Effect of nitrogen sources upon the activity of L-glutamate dehydrogenase of *Lemna gibba*. Phytochemistry 12: 1937-1946.

Smith L., 1955. In: Methods in enzymology. Colowick S. P., Kaplan N. O. (eds.), vol. 2. Academic Press, New York.

Suzuki A., Gadal P., Oaks A., 1981. Intracellular distribution of enzymes associated with nitrogen assimilation in roots. Planta 151: 457-461.

Wewnątrzkomórkowa lokalizacja enzymów metabolizmu azotowego w korzeniach Pisum arvense L.

Streszczenie

Badano zmiany aktywności enzymów związanych z asymilacją azotanów oraz ich wewnątrzkomórkowe rozmieszczenie w korzeniach siewek peluszki (*Pisum arvense L.*). Stwierdzono, że reduktaza azotanowa (EC 1.6.6.2) i syntetaza glutaminowa (EC 6.3.1.2) występują we frakcji cytosolowej, natomiast reduktaza azotynowa (EC 1.6.6.4) i syntaza glutaminianowa (EC 2.6.1.53) związane są z frakcją plastydów. Wyniki wskazują, że system enzymatyczny syntetaza glutaminowa/syntaza glutaminianowa stanowi główny szlak wbudowywania jonów amonowych do związków organicznych w korzeniach siewek peluszki pobierających azotany, natomiast dehydrogenaza glutaminianowa odgrywa mniejszą rolę.