Phytochemistry, 1972, Vol. 11, pp. 2447 to 2449. Pergamon Press. Printed in England.

SHORT COMMUNICATION

ISOELECTRIC FOCUSING OF SPINACH NITRATE REDUCTASE AND ITS TUNGSTEN ANALOGUE

B. A. NOTTON, E. J. HEWITT and A. H. FIELDING

Long Ashton Research Station, University of Bristol, Long Ashton, Bristol BS18 9AF

(*Received* 10 *February* 1972)

Key Word Index—*Spinacea oleracea*; Chenopodiaceae; spinach; nitrate reductase; isoelectric point; isoenzymes.

Abstract—Highly purified nitrate reductase from spinach (*Spinacea oleracea* L.) was submitted to isoelectric focusing. Two peaks of enzyme activity occurred at pH 3·5 and 4·9. Both nitrate reductases could use NADH and reduced benzylviologen but not NADPH as electron donors. The enzymically inactive tungsten analogue of nitrate reductase labelled with ^{185}W during biosynthesis was mixed with the active enzyme before purification and yielded two tungsten protein peaks after isoelectric focusing coincident with those containing nitrate reductase.

INTRODUCTION

ISOELECTRIC focusing has become an important tool for the purification of proteins and for the determination of isoelectric points. However, application of this technique to labile plant enzymes has so far been limited. Recent work[1] has indicated the role of tungsten in the inhibition of nitrate reductase (NR) activity, and in the present paper we offer further proof for the formation of a tungsten analogue of the enzyme and the possible occurrence of two isoenzymes of NR.

RESULTS

The leaves from molybdenum deficient ($-Mo + NO_3$) spinach plants[2] which had been fed with radioactive tungsten and contained the tungsten analogue of NR[1] were mixed with an equal weight of normal ($+Mo + NO_3$) spinach leaves and the NR was purified.[3,4] Table 1 shows the purification of the enzyme up to the alumina C_γ stage and the association of radioactivity and enzyme activity originating from the $-Mo + NO_3 + W$ and the $+Mo + NO_3$ plants respectively.

In early experiments when the partially purified enzyme was subjected to isoelectric focusing in the pH range 5–8, considerable precipitation of protein occurred in the more acid region of the column and some collapse of the bands was found during the 36 hr run; however, two peaks of enzyme activity were consistently detected: the main peak at pH 4·9 and a smaller peak, the location of which varied between pH 2·0 and 3·4.

Further purification of NR by Biogel A-0·5 m filtration on a calibrated column substantially increased the specific activity of the enzyme. The NR and ^{185}W were eluted as a

[1] B. A. NOTTON and E. J. HEWITT, *Biochem. Biophys. Res. Commun.* **18,** 19 (1971).
[2] E. J. HEWITT, in *Sand and Water Culture Methods Used in the Study of Plant Nutrition,* Commonwealth Bureau of Horticulture, Tech. Commun. 22, Commonwealth Agr. Bur., Farnham Royal (1966).
[3] B. A. NOTTON and E. J. HEWITT, *Plant Cell Physiol.* **12,** 465 (1971).
[4] A. PANEQUE and M. LOSADA, *Biochem. Biophys. Acta* **128,** 202 (1966).

single peak corresponding to a MW of approx. 240 000. No bands of precipitated protein were obviously visible during subsequent isoelectric focusing of the desalted eluate.

TABLE 1. PURIFICATION OF NITRATE REDUCTASE FROM EXTRACTS OF $-Mo + NO_3$ SPINACH LEAVES FED WITH ^{185}W MIXED WITH HEALTHY $+Mo + NO_3$ SPINACH LEAVES

Treatment*	Nitrate reductase†	Protein‡	Radioactivity§
Crude extract	1527	19 200	1036
$Ca_3(PO_4)_2$ supern.	111	11 100	549
Pi wash	38	1100	67
PPi eluate	1122	1300	255
0–50% $(NH_4)_2SO_4$ pptn	920	900	204
Alumina C_γ supern.	16	400	8
Dil. PPi wash	3	30	6
PPi eluate	371	400	84

* For details of purification see Refs. 3 and 4.
† Results expressed as nmol NO_2 produced/15 min/g fr. wt.
‡ Results expressed as μg/g fr. wt.
§ Results expressed as c/400 sec/g fr. wt.

As a further precaution against precipitation of the enzyme as an artefact, a layer of 50% sucrose was used to separate the acid electrolyte from the gradient. Two peaks of enzyme activity were consistently found, one at pH 4·9 and the other at pH 3·5, the smaller, more

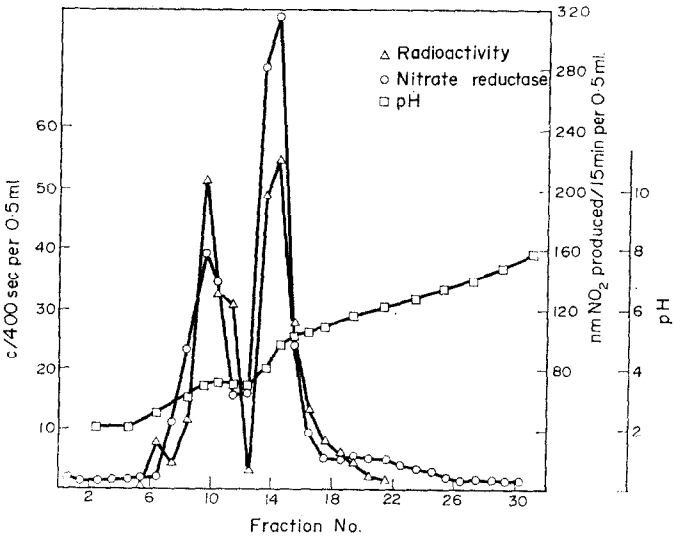

FIG. 1. ISOELECTRIC FOCUSING OF PURIFIED NITRATE REDUCTASE FROM $-Mo + NO_3$ SPINACH LEAVES FED WITH ^{185}W MIXED WITH HEALTHY $+Mo - NO_3$ SPINACH LEAVES. 50 DROP (3·1 ml) FRACTIONS WERE ASSAYED.

acid peak having 25–50% of the NR activity of the other. The ^{185}W radioactivity, which was coincident with enzyme activity throughout the gel filtration stage and with both peaks from the focusing, was almost identical in activity in each peak (Fig. 1). No other peaks of radioactivity were found by either technique.

An attempt to bring both peaks into the ampholyte range by repeating the experiment at pH 3–6 resulted in the 4·9 peak activity being only 19% of the pH 3·5 peak. A similar result was obtained when the pH 4–6 range was used. For both peaks, reduced benzyl-viologen could be used as an alternative electron donor[5] but not NADPH.

DISCUSSION

The results indicate that two isoenzymes of nitrate reductase exist, but apart from their isoelectric points no other differences could be found between them. The difference in peak heights, in terms of enzyme activity and radioactivity indicate some difference in stability, and the reversal of peak heights when lower median pH ampholytes were used may indicate some difference in pH lability. Large losses of enzyme activity occurred during a 36 hr run but any reduction in time resulted in incompletely focused peaks. NR from chlorella and spinach has been reported to have a MW of 500 000;[6] however, a column of agarose (Biogel A-0·5 m) calibrated with myoglobin, ovalbumin, alcohol dehydrogenase, γ-globulin, fibrinogin and thyroglobulin[7] gave an estimated MW for our spinach preparation of 240 000.

The coincidence of the two peaks of radioactivity and enzyme activity through all stages of purification is further evidence for the biosynthesis of a tungsten-containing, inactive protein identical in other respects to NR.

EXPERIMENTAL

Spinach plants, variety Noorman, were grown in sand culture for about 6 weeks with molybdenum-free nutrients ($-Mo + NO_3$) containing a supplement of $0{\cdot}2 \times 10^4$ ppm Mo^3 or with complete nutrient ($+Mo + NO_3$). Biosynthesis of the W analogue of NR was induced in the $-Mo$ plants by the addition of 200 ml of Mo free nutrients containing 40 μCi ^{185}W and 36·4 μg W as WO_4^{2-} for 24–36 hr before sampling.[1] Protein from the leaves was fractionated up to the post alumina C_γ state[3,4] and either, (a) transferred to an 0·01 M Pi, 10^{-3} M EDTA pH 7·5 medium by means of a small Sephadex G25 column, or (b) further purified by gel filtration through a $2{\cdot}5 \times 100$ cm calibrated Biogel A-0·5 m column with 0·1 M Pi, 10^{-3} M EDTA, 10^{-1} M KCl pH 7·5 prior to treatment as in (a). The low ionic strength eluate from the G25 column was then taken as the 'light' solution in the preparation of a 0–50% sucrose gradient for isoelectric focusing in an LKB 8100 apparatus over the required pH range. After 36 hr at 5°, 50 drop fractions were collected for assay. Solutions were tested for NADH-NR,[8] total protein[9,10] only in the early stages when concentrations were sufficiently high and radioactivity was determined by an automated procedure.[11]

Acknowledgements—We thank Mr. C. P. Lloyd-Jones for preparation of radioactive tungsten dilutions and provision of automated counting facilities. We are also grateful to Mr. E. F. Watson for growing the experimental plants, and to Mr. R. J. Fido for technical assistance.

[5] C. F. Cresswell, R. H. Hageman, E. J. Hewitt and D. P. Hucklesby, *Biochem. J.* **94,** 40 (1965).
[6] P. Andrews, *Biochem. J.* **96,** 595 (1965).
[7] A. Ma. Relimpio, P. J. Aparicio, A. Paneque and M. Losada, *FEBS Lett.* **17,** 226 (1971).
[8] E. J. Hewitt and D. J. D. Nicholas, in *Modern Methods of Plant Analysis*, Vol. 7, p. 67, Springer, Berlin (1964).
[9] M. Solacka, J. A. Ross and D. F. Millikan, *Phytochem.* **7,** 1293 (1968).
[10] O. H. Lowry, W. J. Rosebrough, A. L. Farr and R. T. Randall, *J. Biol. Chem.* **193,** 265 (1951).
[11] C. P. Lloyd-Jones and E. J. Skerrett, *Analyst* **94,** 236 (1969).