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ASSAY OF MOLYBDENUM COFACTOR OF BARLEY

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Abstract—Conditions for assay of molybdenum cofactor in barley shoot extracts in the presence of molybdate (25 mM Na_2MoO_4) and the sulphydryl-group protector, reduced glutathione (5 mM), were optimized. Both total Mo-cofactor (assayed after heat-treatment of cell-free extracts) and 'free' Mo-cofactor (assayed in untreated cell-free extracts) were assayed. Compared to control plants grown in the absence of an exogenous nitrogen source total Mo-cofactor levels increased around 70% when plants were grown for 4 days in the presence of either 15 mM KNO_3 or 15 mM NH_4NO_3. Growth in the presence of 15 mM $(NH_4)_2SO_4$ did not affect the Mo-cofactor level. Very similar results were seen when plants were transferred to these nitrogen sources for 24 hr after previous growth in the absence of an exogenous nitrogen source. In contrast, 'free' Mo-cofactor levels of both KNO_3 and NH_4NO_3-treated plants were increased 2–3-fold over untreated controls. Growth in the presence of $(NH_4)_2SO_4$ did not affect the 'free' Mo-cofactor level.

INTRODUCTION

The molybdenum of the higher plant assimilatory nitrate reductase and, with the exception of nitrogenase [1], of all other molybdo-enzymes so far examined is carried on a dissociable, dialysable, oxygen sensitive moiety, the molybdenum cofactor (Mo-cofactor) [1–3]. This cofactor has recently been shown to contain a novel pterin [4] and it has been proposed that the Mo-cofactor is a complex between this pterin (molybdopterin) and molybdenum [5, 6]. Molybdopterin is also able to supply the thiol ligands of Mo detected in several molybdo-enzymes [5, 6].

Several higher plant mutants altered in the pathway leading to synthesis of a functional Mo-cofactor have been isolated and, in some cases, characterized. In *Nicotiana* species three loci, *cnx*A [7–9], *cnx*B [10, 11] and *cnx*C [12, 13] have been described [14]. The biochemical characteristics of these mutants suggest that the *cnx*A locus is involved in insertion of Mo into the molybdopterin, that the *cnx*B locus is involved in the intracellular processing of Mo/molybdopterin and that the *cnx*C locus is involved in the synthesis of the molybdopterin.

The Mo-cofactor may be assayed by restoration of NADPH-nitrate reductase (EC 1.6.6.2) activity in extracts of *Neurospora crassa* mutant *nit*-1 (which is believed to lack the molybdopterin moiety of Mo-cofactor) and depends on the dimerisation of NADPH-nitrate reductase haemoflavoprotein subunits in the presence of exogenous Mo-cofactor [15]. Using this assay, Mo-cofactor activity has been determined in extracts of wild-type and mutant tobacco callus tissue [8, 11, 16] after release of Mo-cofactor from protein carriers by either acid [17] or, more effectively, heat treatment [18, 19]. The results of these studies have been interpreted to show that the synthesis of Mo-cofactor and nitrate reductase, at least in tobacco, have a common regulatory mechanism and that nitrogen nutrition affected Mo-cofactor levels [16].

Several conditional-lethal *cnx*-type barley mutants have been isolated recently and are being characterized [20; B. Steven, S. W. J. Bright and J. L. Wray, unpublished]. In this paper we report conditions for the assay of Mo-cofactor in extracts of wild-type barley. The effect of nitrogen nutrition on the levels of Mo-cofactor was also examined.

RESULTS AND DISCUSSION

Conditions for assay of Mo-cofactor were optimized using heat-treated cell-free extracts of barley shoots. Heat treatment is believed to release Mo-cofactor from a 'bound' form and make it available for assay. The 'bound' form of Mo-cofactor presumably includes Mo-cofactor bound to enzymes such as nitrate reductase and xanthine dehydrogenase which are present in barley [20, 21] as well as perhaps to carrier proteins. Also with barley, heat release of Mo-cofactor turned out to be an efficient and reproducible method. The optimum for release of Mo-cofactor in the presence of 25 mM sodium molybdate and 5 mM reduced glutathione was found to be 90 sec at 80° (Fig. 1). Glutathione, at 5 mM, was a much better protector of Mo-cofactor activity than either cysteine, dithiothreitol or the previously used β-mercaptoethanol [19] (Fig. 2 and Table 1). Ascorbic acid, which had been used previously with β-mercaptoethanol to protect Mo-cofactor activity [19] gave lower Mo-cofactor activities in the presence of glutathione and was therefore omitted. Mo-cofactor activities were higher if heating was carried out under nitrogen (Table 1). A complementation time of 40 min and a buffer to tissue ratio of 10:1 (Fig. 3) were

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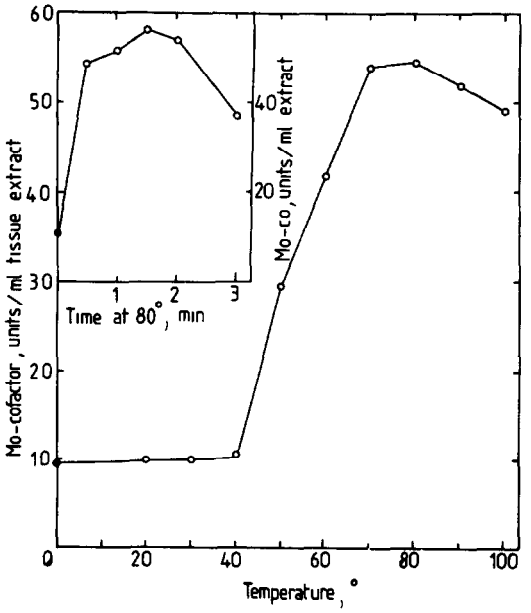

Fig. 1. Effect of temperature pretreatment on Mo-cofactor activity of extracts from barley. Barley seedlings grown on KNO_3 as N-source were extracted as described in Experimental. Aliquots were taken, incubated for 90 sec at the temperatures indicated and tested for Mo-cofactor activity. Inset: Effect of duration of incubation at 80° on Mo-cofactor activity. Aliquots were withdrawn at the times indicated and tested for Mo-cofactor activity.

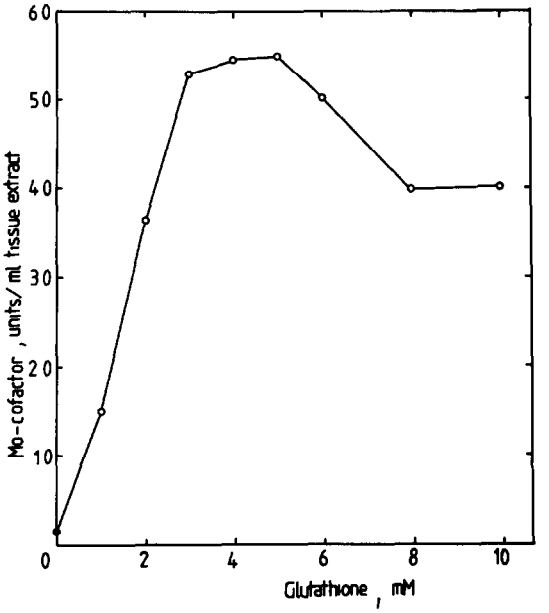

Fig. 2. Effect of reduced glutathione on the Mo-cofactor activity of barley. Barley seedlings grown on KNO_3 as N-source were extracted, heat-treated and tested for Mo-cofactor activity as described in the Experimental, with the exception that the extraction buffer contained the indicated concentrations of reduced glutathione.

found to be optimal for assay of Mo-cofactor activity.

The optimized conditions above were used to determine the level of total assayable Mo-cofactor (determined after heat-treatment) and of 'free' Mo-cofactor (determined without a heat treatment) (Table 2)*. Both 'free' Mo-cofactor and 'bound' Mo-cofactor (determined as the difference between total and 'free' Mo-cofactor) were present in cell-free extracts of barley shoots which had not been given an exogenous nitrogen source, but 'free' Mo-cofactor was only around 18% of the 'bound' Mo-cofactor level. Growth in the presence of $(NH_4)_2SO_4$ for 4 days did not adversely affect the level of Mo-cofactor. Both 'free' and 'bound' Mo-cofactor, as well as nitrate and nitrate reductase levels, increased when plants were grown in the presence of either NH_4NO_3 or KNO_3 for 4 days compared to controls. 'Free' Mo-cofactor levels increased 2–3-fold whilst 'bound' Mo-cofactor increased by around 65%. There was an overall increase in total assayable Mo-cofactor of around 73%. Very similar results, both in trends and absolute activities, were obtained when plants were grown for 4 days without an exogenous nitrogen source and then treated with either NH_4NO_3 or KNO_3 for 24 hr before assay (data not shown).

These results show that total Mo-cofactor levels are increased in response to nitrate nutrition. This phenomenon has been reported previously for tobacco callus cells [16]. A 73% increase in total Mo-cofactor in response to nitrate nutrition may not seem very great, particularly if one recalls that nitrate reductase levels may increase very many fold in response to nitrate. However in the absence of nitrate, plants possess no nitrate reductase activity (Table 2) and hence the Mo-cofactor levels assayed before nitrate addition are unrelated to nitrate reductase and presumably reflect Mo-cofactor present as part of other molybdoenzymes or molybdenum storage proteins

Table 1. The relative effectiveness of sulphydryl group protecting reagents and of anaerobic conditions on the assay of Mo-cofactor of barley

Sulphydryl group protecting reagent	Anaerobic conditions	Mo-cofactor activity: nmol/ml tissue extract	Mo-cofactor activity: %
Glutathione (5 mM)	+	34.8	100
	−	33.1	95
Cysteine (15 mM)	+	0.95	2.7
	−	0.62	1.8
Dithiothreitol (7.5 mM)	+	8.84	25
	−	1.95	5.6
Mercaptoethanol (12 mM)	+	2.59	7.4
	−	2.11	6.1

Mo-cofactor was assayed as described in Experimental. The concentrations of sulphydryl group protecting reagents used were previously determined to be optimal. The seedlings were grown on KNO_3 (15 mM) as N-source.

*The term 'free' does not necessarily mean that Mo-cofactor appears as the free moiety but describes a state in which the Mo-cofactor is assayable by the *nit*-1 complementation assay.

Table 2. Effect of nitrogen nutrition on molybdenum cofactor, nitrate and nitrate reductase levels of 4-day old barley

Treatment	Mo-cofactor activity (units/mg protein) with heat treament	Mo-cofactor activity (units/mg protein) without heat treament	Nitrate content (μmol/g)	Nitrate reductase activity (μmol/mg protein/hr)
No nitrogen source	30.5	4.6	0	0
15 mM $(NH_4)_2SO_4$	30.3	3.5	0	0
15 mM NH_4NO_3	50.1	11.5	17.7	0.48
15 mM KNO_3	55.4	12.7	23.3	0.44

Mo-cofactor, nitrate and nitrate reductase levels were determined as in the Experimental. Results are representative of between three and five separate determinations.

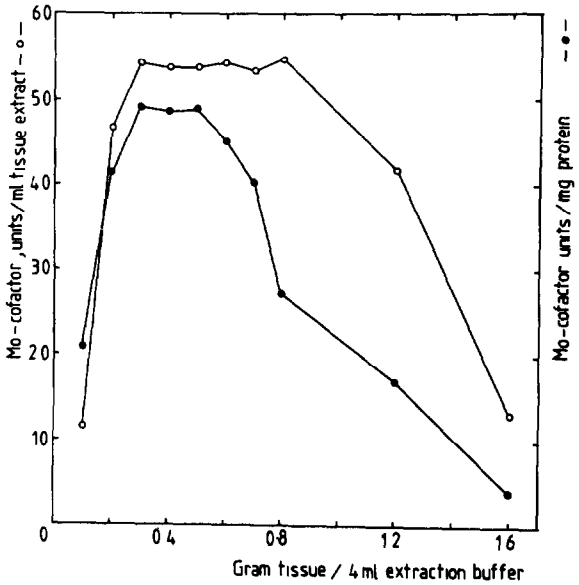

Fig. 3. Influence of the buffer-to-tissue ratio on the Mo-cofactor activity of barley. Barley seedlings grown on KNO_3 as N-source were extracted at the indicated buffer-to-tissue ratios, heat-treated and tested for Mo-cofactor activity as described in Experimental. Absolute and specific activities are given.

('bound' Mo-cofactor) or available to them ('free' Mo-cofactor). Nitrate brings about a measurable increase of 20 Mo-cofactor units under the conditions used here which probably represents both Mo-cofactor as a constituent of nitrate reductase (i.e. 'bound' Mo-cofactor) and Mo-cofactor available for incorporation into new nitrate reductase molecules ('free' Mo-cofactor). Both 'bound' and 'free' Mo-cofactor increase in response to nitrate nutrition. Ammonium ions have no effect on what may be termed the constitutive Mo-cofactor level (before nitrate addition) nor on the nitrate promoted increase in Mo-cofactor level (Table 2).

EXPERIMENTAL

Growth of plant material. Seeds of barley, *Hordeum vulgare* L. cv. Golden Promise, were sown in vermiculite and treated with the appropriate modified half-strength Hoagland nutrient solution. Plants were maintained in the dark at 28° for 3 days during which time the vermiculite was kept moist by adding the same known vol. of the appropriate soln. At 3 days, plants were transferred into the light at 28°. Plants were treated with modified half-strength Hoagland nutrient soln either lacking a nitrogen source or containing either 15 mM KNO_3, 15 mM $(NH_4)_2SO_4$ or 15 mM NH_4NO_3 as sole nitrogen source. After transfer into the light at 3 days plants were harvested 24 hr later and assayed.

Extraction of tissue. Barley shoots (0.4 g) were excised above the seed and ground in a mortar and pestle in 4 ml of 50 mM KPi buffer, pH 7.5, containing 0.5 mM EDTA, 25 mM sodium molybdate and 5 mM reduced glutathione. The brei was centrifuged at 25 000 *g* for 20 min at 4° and the supernatant was used for determination of molybdenum cofactor activity, nitrate reductase activity, and nitrate and protein content.

Assay of NADH-nitrate reductase. NADH-nitrate reductase activity was assayed by the method of ref. [21].

Nitrate assay. Nitrate was assayed by the method of ref. [10].

Protein assay. Protein was assayed by the method of ref. [22] using BSA as standard.

Molybdenum-cofactor assay. The cell-free extract was evacuated and flushed with N_2, stoppered and heated for 90 sec at 80°. The extracts were cooled on ice and centrifuged if necessary. Mo-cofactor source (50 μl) and 100 μl of *nit*-1 extract containing 1 mM NADPH were mixed, evacuated, flushed with N_2 and incubated for 40 min at 25°, the optimal time for complementation. NADPH-nitrate reductase activity was assayed as described [19]. One unit of Mo-cofactor activity will reconstitute the *nit*-1 nitrate reductase to 1 nmol/min. Sp. act. is expressed as units/mg protein of Mo-cofactor source.

N. crassa nit-1 *extract.* This was prepared as described [19].

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