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RELEASE OF MOLYBDENUM CO-FACTOR FROM NITRATE REDUCTASE AND XANTHINE OXIDASE BY HEAT TREATMENT

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; molybdenum co-factor; nitrate reductase; xanthine oxidase; co-factor release; heat treatment; *in vitro* complementation.

Abstract—Heat treatment (90 sec at 70°) is shown to convert the bound molybdenum co-factor of tobacco cell-free extracts and bovine milk xanthine oxidase into a form capable of complementing the *Neurospora crassa* mutant *nit*-1. In the presence of 1 mM ascorbic acid, 25 mM molybdate and, for plant extracts, sulphydryl group protecting agents, the molybdenum co-factor can survive incubations up to 100° whilst maintaining its biological activity. Especially with plant extracts, the efficiency of heat treatment is considerably higher than that of the acidification procedure which is often utilized for releasing molybdenum co-factor.

INTRODUCTION

Molybdenum co-factor is a low-MW, non-covalently bound constituent of several molybdo-enzymes [1]. To release molybdenum co-factor from purified molybdo-enzymes [2–4] or cell-free extracts [5–7], an acidification procedure (1–5 min at pH 2–2.5) followed by neutralization is frequently used. Recently it has been shown for cell-free extracts of tobacco cell cultures, where regulation of molybdenum co-factor metabolism is tightly connected to nitrate reductase [$NADH_2$: nitrate oxidoreductase, EC 1.6.6.1] [8], and for milk xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2) [4], that acid treatment is not sufficient to achieve a quantitative release of molybdenum co-factor. Therefore, subsequent to acidification, cell extracts were subjected to a short heat treatment (7 min at 80°) which gave rise to a several-fold higher co-factor activity than that achieved by acid treatment alone [4, 8].

Acidification–neutralization causes dilution of molybdo-enzyme samples and does not allow the assay of larger numbers of samples (e.g. fractions of column eluates or density gradients) in a strictly reproducible manner. I here report a procedure for releasing molybdenum co-factor from plant cell-free extracts and purified milk xanthine oxidase which omits the acid treatment step and replaces it with a simple, quick and reproducible heat treatment step which gives molybdenum co-factor activities superior or similar to the acidification method. Very recently [9], heat treatment has also been shown to be effective in releasing molybdenum co-factor from prokaryotic sources.

Molybdenum co-factor activity was assayed by restoration of nitrate reductase activity in *Neurospora crassa* mutant *nit*-1 extracts. NADPH-nitrate reductase activity was taken as a measure for the amount of biologically active molybdenum co-factor.

RESULTS AND DISCUSSION

The widely used acidification step for release of bound molybdenum co-factor could be omitted and completely replaced by simply heating the sample. Figure 1 shows the temperature dependence and time course of release of molybdenum co-factor from tobacco cell culture extracts and milk xanthine oxidase. Starting at 50–60° the temperature induced release of molybdenum co-factor rises steeply to reach a maximum at 70–75°, thereafter decreasing slightly. Heat treatment can be assumed to cause drastic conformational changes in co-factor-containing proteins (molybdo-enzymes, carrier-proteins) leading ultimately to the release of co-factor. The very high stability

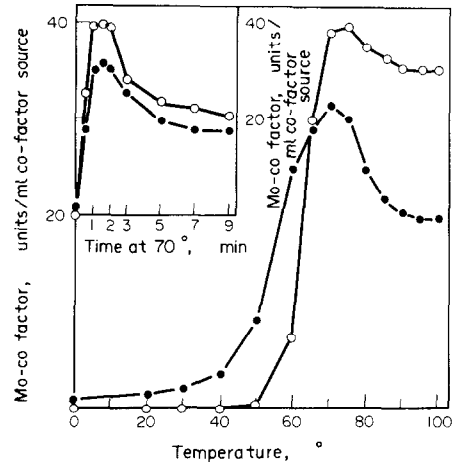

Fig. 1. Effect of temperature pretreatment on molybdenum co-factor activity of tobacco callus extract and milk xanthine oxidase. Tobacco callus (●) was extracted in buffer A. Milk xanthine oxidase (○) was dissolved in buffer B. Aliquots were taken, incubated for 90 sec at the temperatures indicated and tested for molybdenum co-factor activity. Inset. Effect of duration of incubation at 70° on molybdenum co-factor activity. Aliquots were withdrawn at the times indicated and tested for molybdenum co-factor activity. Protein concentration: tobacco callus, 1.9 mg/ml; xanthine oxidase, 0.1 mg/ml.

of the co-factor at elevated temperatures provides a further indication for the nonprotein nature of the structural moiety of molybdenum co-factor, recently identified as a novel pteridine [15]. All the following experiments were performed under standard heating conditions of 90 sec at 70°.

To obtain high molybdenum co-factor activities, it was necessary to include ascorbic acid and molybdate in the buffer that was used for cell extraction (when testing crude extracts) or for dissolving the purified molybdo-enzymes (Table 1). Ascorbic acid, which was previously shown to protect molybdenum co-factor activity during heat treatment [4, 8], was optimal at a concentration of 1–2 mM. The addition of 25 mM molybdate to the heat treatment buffer (resulting in a final concentration of 8 mM in the *nit*-1 complementation mixture), enhanced molybdenum co-factor activities 10–20-fold. Molybdenum is known to be rapidly lost from the released co-factor [3, 4, 9]. Hence, the requirement for 5–10 mM molybdate in the *nit*-1 complementation mixture is not an unusual observation but a characteristic common to assays of molybdenum co-factor from diverse phylogenetic sources ranging from bacteria [4, 6, 9], fungi [3] and plants [5, 8] to mammals [3, 4]. In the presence of ascorbic acid and molybdate, the half-life at 4° of heat-released tobacco molybdenum co-factor was *ca* 4 hr.

For plant cells, but not for milk xanthine oxidase and *Neurospora* mycelia, it was essential to add a sulphydryl group protecting agent to the extraction buffer (Table 1). Mercaptoethanol (12 mM) or dithiothreitol (5 mM) were optimal for tobacco callus. This requirement for sulphydryl group protectors during heat treatment provides an explanation for the suboptimal results obtained previously [8] for tobacco cells after heat treatment in the absence of mercaptoethanol.

Table 2 shows, for samples of plant, fungal, and mammalian origin, a comparison of the efficiencies of molybdenum co-factor release achieved by conventional acidification and by the new heat treatment procedure. It can be seen that heat treatment is especially useful for plant samples where acid treatment is not sufficient to achieve complete release of molybdenum co-factor. With milk xanthine oxidase both methods gave similar results, and with crude extract of NH_4^+-grown *Neurospora*, no releasing treatment seems to be necessary.

Thus, in plant cells, 90–95% of the total amount, of molybdenum co-factor exists in a bound form that, in the absence of a releasing treatment, is not able to complement *nit*-1 (Table 1). In contrast, in NH_4^+-grown *Neurospora*, molybdenum co-factor seems to exist exclusively in a form able to complement *nit*-1 which, however, does not necessarily mean that this form is the free, unbound one.

Since heat treatment is a strictly reproducible method that can be performed much faster and easier than acidification–neutralization, it is particularly applicable for the simultaneous processing of large numbers of samples obtained after CC and density gradient centrifugation. Figure 2 shows the elution profile of milk xanthine

Table 1. Influence of extraction buffer on molybdenum co-factor activity after heat treatment

Extraction buffer	Mo co-factor activity (units/mg protein)		
	Tobacco callus	Xanthine oxidase	*Neurospora* wild type
PE	0.5	4	0.5
PE/Asc/Mo	0.8	390	14.0
PE/M	0.5	22	0.5
PE/M/Asc	0.8	30	0.6
PE/M/Mo	5.8	113	4.7
PE/M/Asc/Mo	16.0	253	8.4

Molybdenum co-factor sources were extracted or dissolved in the indicated buffers and incubated for 90 sec at 70° (P = phosphate; E = EDTA; M = mercaptoethanol; Asc = ascorbic acid; Mo = sodium molybdate). All concentrations are as described in Experimental. Mean values of three experiments are given. Protein concentration of extracts: tobacco callus (1.5–2.5 mg/ml), milk xanthine oxidase (0.1 mg/ml), *Neurospora* (6–8 mg/ml). In the absence of *nit*-1 extract, no NADPH-nitrate reductase activity could be detected.

Table 2. Effect of pretreatment on molybdenum co-factor activity from different sources

Co-factor source	Mo co-factor activity (units/mg protein)		
	No treatment	1 min at pH 2.3	90 sec at 70°
Tobacco callus	0.6	2.8	13.0
Xanthine oxidase	0	366	393
Neurospora wild type	14.0	13.5	14.0

Molybdenum co-factor sources were extracted in buffer A (tobacco) or buffer B (xanthine oxidase, *Neurospora*) and tested for molybdenum co-factor activity before (= no treatment) and after acidification and heat treatment, respectively. Mean values of three experiments are given. Protein concentrations were as in the legend to Table 1. In the absence of *nit*-1 extract, no NADPH-nitrate reductase activity could be detected.

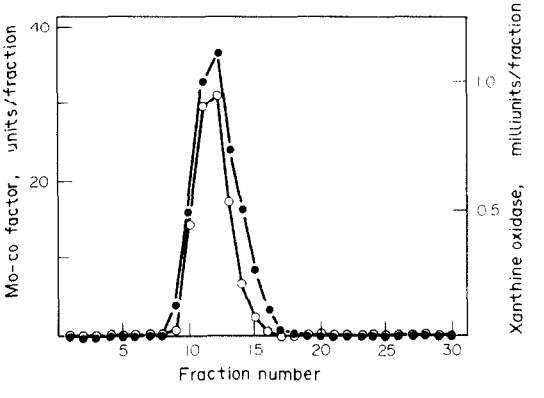

Fig. 2. Sephadex G-25 gel filtration of milk xanthine oxidase. A solution (0.3 ml) of milk xanthine oxidase (3 mg) was applied to a column (2.5 × 25 cm) of Sephadex G-25 equilibrated in 50 mM Pi buffer, pH 8.0. Fractions (1 ml) were collected at room temperature. Xanthine oxidase activity (○) was determined as described. Aliquots of the fractions were withdrawn, made 1 mM and 25 mM in ascorbic acid and sodium molybdate, respectively, heated for 90 sec at 70° and tested for molybdenum co-factor activity (●).

oxidase after gel filtration. The activity profile of molybdenum co-factor matches perfectly that of xanthine oxidase activity.

EXPERIMENTAL

Cells and culture conditions. The wild type cell line S21-1 of amphi-haploid *Nicotiana tabacum* var. Gatersleben was maintained as a callus culture on agar medium containing NO_3^- and NH_4^+ as nitrogen source as described previously [10, 11]. *Neurospora crassa* wild type and mutant strain *nit*-1 were grown on NH_4^+ as described previously [5], with the exception that with *nit*-1 the time for nitrate induction was increased to 5 hr. *N. crassa* wild type was used uninduced.

Cell extraction. Tobacco callus tissue (0.5 g) was broken-up in 2 ml buffer A (50 mM K/NaPi, pH 7.5, 0.5 mM EDTA, 12 mM mercaptoethanol, 1 mM ascorbic acid, 25 mM Na_2MoO_4) by treatment for 60 sec at maximal output with the microtip of a Branson B-12 sonifier under efficient cooling with crushed ice. After centrifugation at 4° for 20 min at 20000 *g*, the supernatant was used as the source of Mo co-factor. Preparation of cell-free extracts from *Neurospora nit*-1 was as described previously [5]. Since preliminary expts (not shown) corroborated the previous finding [3] that 50 mM Pi buffer gave higher Mo co-factor activities than 100 mM Pi, this lower concn was used also in the *nit*-1 extraction buffer. *N. crassa* wild type cells were broken under liquid N_2 with a mortar and pestle and resuspended in four vols. (w/v) buffer B (as A, but mercaptoethanol omitted). Bovine milk xanthine oxidase (Sigma, Munich) (10 μl) was dissolved in 1 ml buffer B.

Heat treatment. The extract was thoroughly evacuated, flushed with N_2, stoppered, incubated for 90 sec at 70°, and immediately cooled on ice. Ppted protein was removed by centrifugation.

Acid treatment. Cells of tobacco or *Neurospora* were extracted as described above in four vols. (w/v) acid treatment buffer (0.1 M NaCl, 1 mM ascorbic acid, previously adjusted to pH 2.0 with HCl). The homogenate was readjusted to pH 2.3 with a few drops of 1 M HCl, maintained at this pH for 1 min and neutralized with 25 % vol. of 0.2 M K/NaPi, pH 7.5. After adding Na_2MoO_4 to a final concn of 25 mM, the homogenate was centrifuged as above. Xanthine oxidase (10 μl) was dissolved in 0.6 ml acid treatment buffer, neutralized after 1 min at room temp. with 0.3 ml 0.2 M Pi (pH 7.5) and made 25 mM with respect to Na_2MoO_4.

Restoration and assay of nit-1 *nitrate reductase.* 100 μl *nit*-1 extract, 50 μl Mo co-factor and 5 μl NADPH (20 mM) were mixed, evacuated, flushed with N_2 stoppered and incubated for 1 hr at 25°. The complementation was complete after *ca* 30–50 min. With 100 μl *nit*-1 extract, the assay was linear up to 60 μl of Mo co-factor. Subsequently, 100 μl FAD (0.1 mM) and 50 μl KNO_3 (0.1 M) were added and incubation was continued for 15–30 min. The reaction was stopped by boiling the assay mixture and nitrite was determined as described in ref. [12]. The nitrate reductase test was linear up to 30–45 min. One unit of Mo co-factor activity will reconstitute the *nit*-1 nitrate reductase to 1 nmol/min. Sp. act. is expressed as units per mg protein of Mo co-factor extract. Protein was measured by the method of ref. [13] with bovine serum albumin as standard. Activity of xanthine oxidase was determined according to ref. [14] on the basis of its diaphorase activity with cytochrome *c* as acceptor.

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