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Purification and characterization of NAD(P)H quinone reductase from the latex of *Hevea brasiliensis* Müll.-Arg. (Euphorbiaceae)

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Abstract

NAD(P)H quinone reductase [NAD(P)H–QR] present in the latex of *Hevea brasiliensis* Müll.-Arg. (Euphorbiaceae) was purified to homogeniety from the B-serum fraction obtained by freeze-thawing of the bottom fraction of ultracentrifuged fresh latex. The purification protocol involved acetone fractionation, heat treatment, ion exchange chromatography and affinity chromatography. The M_r determined by SDS–PAGE for the protein subunit was 21 kDa, and the molecular mass of the native enzyme estimated by gel filtration was 83 kDa, indicating that the native enzyme is a homotetramer. The enzyme showed pH stability over a range of 6 to at least 10 (with an optimum at pH 8) and thermal stability up to 80 °C. High NAD(P)H–QR activity (70%) was still retained after 10 h of preincubation at 80 °C. A comparable substrate specificity for this enzyme was observed among menadione, *p*-benzoquinone, juglone, and plumbagin, with only duroquinone generating a lower activity. Positive correlations between latex NAD(P)H–QR activity and rubber yield per tapping [fresh latex ($r=0.89$, $P<0.01$), dry rubber ($r=0.81$, $P<0.01$)] together with flow time ($r=0.85$, $P<0.01$) indicated that enzyme activity could possibly be used as a marker to predict the yield potential of selected clones.

Keywords: *Hevea brasiliensis*; Euphorbiaceae; Rubber latex; NAD(P)H quinone reductase

1. Introduction

Plant NAD(P)H quinone reductase [NAD(P)H–QR] uses either NADH or NADPH as the electron donor and a hydrophilic quinone as the electron acceptor. This enzyme is usually present in the soluble cell fraction (Guerrini et al., 1994; Trost et al., 1995; Sparla et al., 1996), but certain NAD(P)H-dependent duroquinone reductase activities may also be found in the mitochondria, microsomes and plasma membranes (Asard et al., 1987; Luster and Buckhout, 1989; Valenti et al., 1990; Luethy et al., 1991; Serrano et al., 1994; Rescigno et al., 1995). It has been found that NAD(P)H–QR purified from a variety of plant tissues comprises subunits in the range of 21.4–27 kDa (Trost et al., 1995, 1997; Luster and Buckhout, 1989; Sparla et al., 1996). These subunits bind flavin mononucleotide as a specific prosthetic group and upon aggregation are arranged into active catalytic homotetramers (Sparla et al., 1996). NAD(P)H–QR appears to be the only soluble plant-cell enzyme that produces fully reduced quinols, without semiquinone intermediates, thereby preventing the build up of the active oxygen species that result from semiquinone autooxidation (Trost et al., 1995). Its metabolic pathway is similar to that of DT-diaphorase found in animal cells (Prochaska et al., 1985; Tedeschi et al., 1995). The quinols produced as a result of the enzyme activity are relatively stable compounds that can be detoxified in plant cells and stored as conjugates in vacuoles (Harborne, 1980).

In this paper we describe the purification and characterization of NAD(P)H–QR extracted from the latex of *Hevea brasiliensis* Müll.-Arg. (Euphorbiaceae) and the correlation between enzyme activity and latex production.

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2. Results and discussion

2.1. Purification of latex NAD(P)H–QR

NAD(P)H–QR was purified from latex B-serum (obtained as described in Experimental) according to the protocol summarized in Table 1. The purification protocol included acetone fractionation, heat treatment and DEAE-Sephacel chromatography, followed by a final purification step on Blue Sepharose CL-6B affinity chromatography to produce a homogeneous enzyme. The final step facilitated a 409-fold increase in purification to give an enzyme with a specific activity of 1.23 μkat mg^{-1} (Table 1).

2.2. Molecular weight of latex NAD(P)H–QR

The M_r determined by SDS-PAGE for the protein subunit was 21 kDa (Fig. 1), and the molecular mass for the native enzyme estimated from gel filtration was 83 kDa (Fig. 2). It thus seems that the 83-kDa fraction is composed of four identical 21-kDa monomer homosubunits (Fig. 1). The earlier-described molecular masses are in the same range as those reported for the enzyme in sugar beet cells (Trost et al., 1995), zucchini hypocotyls (Trost et al., 1997) and tobacco leaves (Sparla et al., 1996).

2.3. Effect of pH and temperature on activity and stability of latex NAD(P)H–QR

Latex NAD(P)H–QR exhibited stability over a broad pH range (6–10, or even higher), with a sharp optimum at pH 8 (Fig. 3). The enzyme exhibited good thermal stability up to 80 °C, with little loss of enzyme activity in the incubation mixture (Fig. 4A). More than 80 and 70% of the latex NAD(P)H–QR activity was retained after 10 h of preincubation at 70 and 80 °C, respectively (Fig. 4B), indicating the unusually high thermal stability as compared to pea seed NAD(P)H–QR which lost 95% activity at 50 °C within 5 min (Wosilait and Nason, 1954).

2.4. Substrate specificity of latex NAD(P)H–QR

Maximal activity was obtained when NADH was used as the electron donor and a hydrophilic short-chain quinone, such as menadione or *p*-benzoquinone, as the acceptor (Table 2). However, there was little difference on substrate specificity among the four quinone substrate

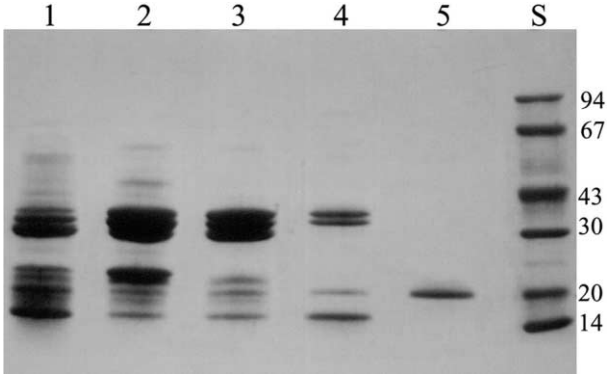

Fig. 1. SDS–PAGE analysis of the purified latex NAD(P)H–QR. Lane 1: B-serum (100 μg); lane 2: 30–50% acetone pellet fraction (100 μg); lane 3: as 2 but after heat-treatment (50 μg); lane 4: peak fraction from DEAE-Sephacel column (15 μg); lane 5: peak fraction of Blue Sepharose CL-6B column (10 μg); lane S: Standard protein markers (kDa).

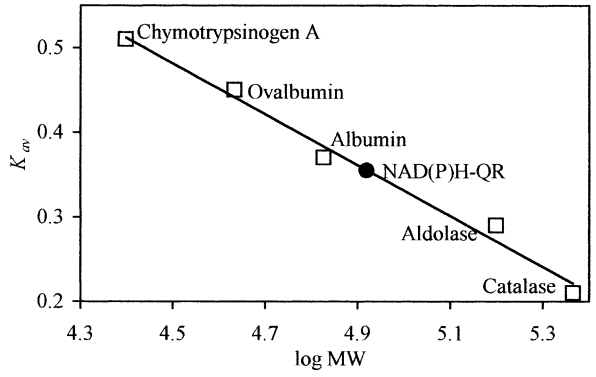

Fig. 2. Calibration curve for determination of the molecular weight of the native NAD(P)H–QR after a Sephadex G-200 column chromatography. The black circle indicates K_{av} of the NAD(P)H-QR with M_r corresponding to 83 kDa.

Table 1
Purification protocol for latex NAD(P)H–QR

Purification step	Total activity (μkat)	Protein (mg)	Specific activity (μkat mg^{-1})	Yield (%)	Purification (-fold)
B-serum	8.07	2717	0.003	100	1
Acetone precipitation	5.25	634	0.008	65	3
Heat treatment	4.32	308	0.014	54	5
DEAE-Sephacel	3.33	25.5	0.131	41	43
Blue Sepharose	1.35	1.1	1.227	17	409

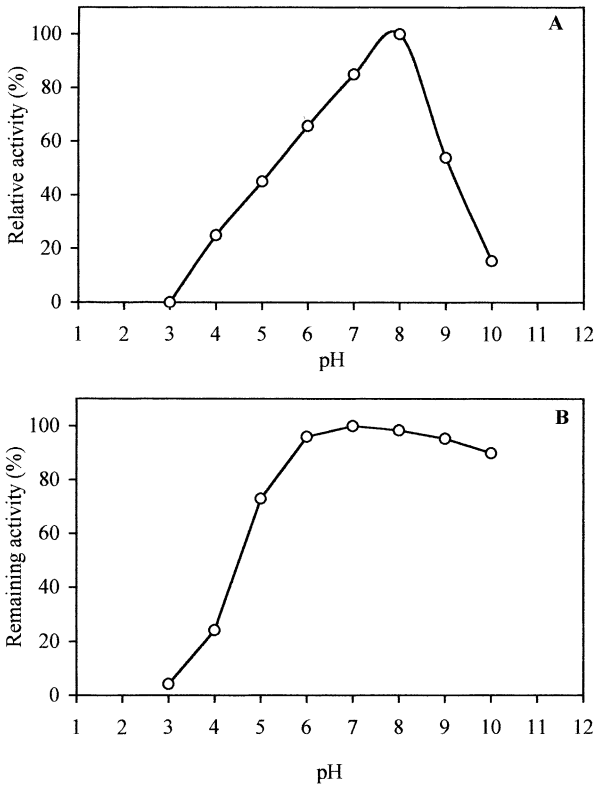

Fig. 3. pH optimum (A) and pH stability (B) of Blue Sepharose-purified NAD(P)H–QR.

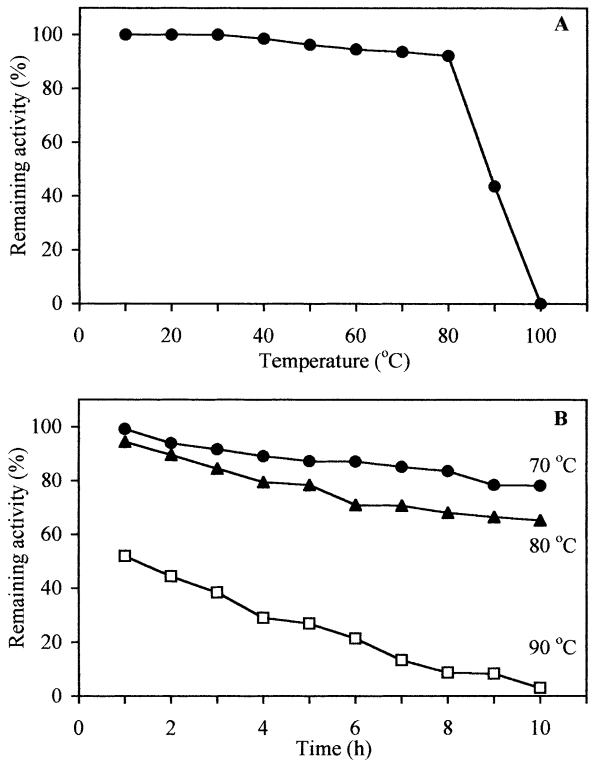

Fig. 4. Thermal stability of Blue Sepharose-purified NAD(P)H–QR. The enzyme was preincubated at different temperatures for 30 min before being assayed (A). The enzyme was preincubated at different temperatures for up to 10 h and was assayed at different points during the time course (B).

acceptors (menadione, *p*-benzoquinone, juglone, and plumbagin), though a slightly lower level was noticeable on duroquinone. A comparable K_m for NADH and NADPH was observed, using any respective quinone acceptors, except for menadione where a lower level was found towards NADH (Table 2). The K_m values (0.2–0.4 mM) of latex NAD(P)H–QR are in the same range as those reported for tobacco leaves (Sparla et al., 1996) but several fold higher than onion root plasma membrane (Serrano et al., 1994). The results of this study thus showed low substrate specificities of latex NAD(P)H–QR on either the acceptor (quinone) or electron donor [NAD(P)H] substrates.

Table 2
Specificity of donor and acceptor substrates for purified NAD(P)H–QR[a]

Acceptor	Donor (μkat mg^{-1})		$K_{m\ (donor)}$ (μM)	
	NADH	NADPH	NADH	NADPH
Menadione	0.562	0.377	204	322
p-Benzoquinone	0.555	0.380	333	384
Juglone	0.542	0.368	250	286
Plumbagin	0.523	0.350	200	270
Duroquinone	0.317	0.250	375	435

[a] Assays were performed in 50 mM Tris–HCl, pH 8, in the presence of 0.2 mM pyridine nucleotide, 0.05 mM of each quinone, as indicated, and 10 μg of purified NAD(P)H–QR (after Blue Sepharose CL-6B column).

2.5. Correlations between levels of NAD(P)H–QR activity and rubber flow time, dry rubber concentration and flow time and between flow time and fresh latex and dry rubber per tapping

The correlation between NAD(P)H–QR activity in the latex and the dry rubber content was investigated with the aim of establishing the effect of the enzyme on latex flow time and hence on latex yields. A positive correlation was observed between NAD(P)H–QR activity in the latex and flow time per tapping ($r = 0.85$, $P < 0.01$), but not between the latter and latex rubber concentration ($r = -0.61$, $P < 0.01$) (Fig. 5). This finding may suggest a supportive role of NAD(P)H–QR in stabilization of the colloidal latex through its action in maintaining particle stability, particularly the integrity of the lutoid membrane. The contribution of the enzyme reaction to latex stability may be attributed to the fact that it yields fully reduced quinols and not semiquinone intermediates, which can cause the build up of active oxygen species, leading to latex destabilization and hence vessel plugging. This type of catalytic activity is similar to that reported for the enzyme in sugar beet

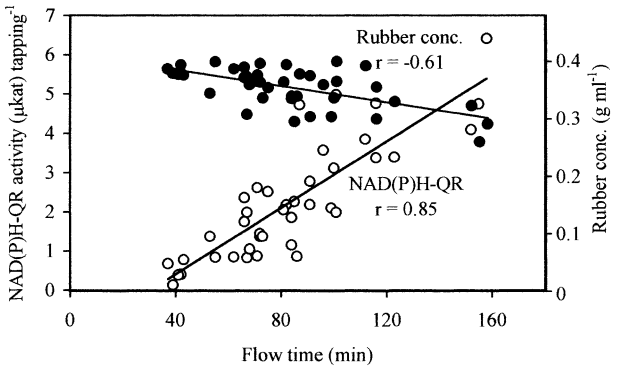

Fig. 5. Correlation between latex NAD(P)H–QR activity and flow time (○) and between latex dry rubber concentration and flow time (●) per tapping ($n=40$).

cells (Trost et al., 1995) or for DT-diaphorase in animal cells (Prochaska et al., 1985; Tedeschi et al., 1995). Finally, the higher the NAD(P)H–QR activity, the longer the flow times of the latex per tapping and hence the higher yields (Fig. 6). The practical implication of our results is that this latex enzyme activity may be used as an indicator of potential rubber yields in the early selection of superior seedlings with improved rubber yields.

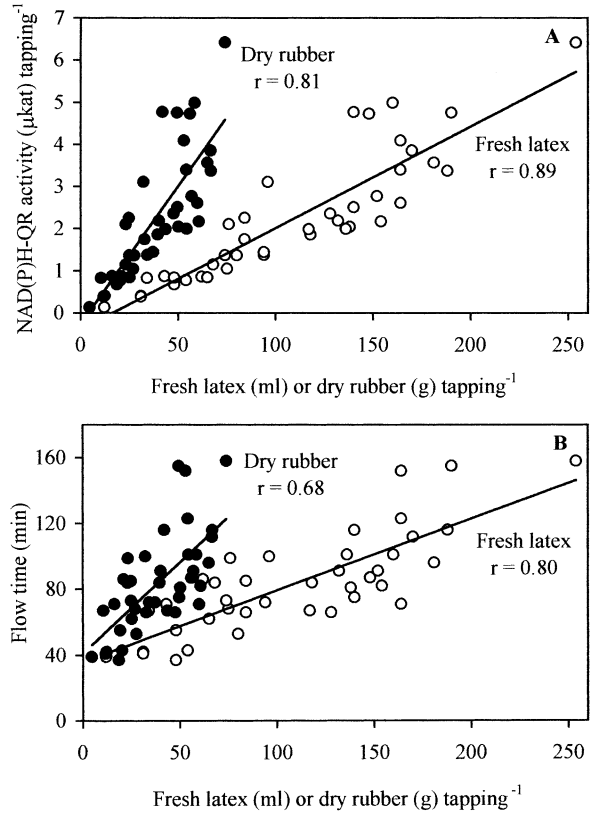

Fig. 6. Correlation between rubber yield and NAD(P)H–QR activity (A) or flow time (B) per tapping ($n=40$).

3. Experimental

3.1. Chemicals

Pyridine nucleotides (NADH, NADPH), menadione, *p*-benzoquinone, plumbagin, juglone, DEAE-Sephacel, Sephadex G-200, Blue Sepharose CL-6B were obtained from Sigma. All other chemicals were of reagent grade.

3.2. Plant material (collection and fractionation of latex)

Freshly tapped latex was collected in an ice-chilled beaker from regularly tapped *H. brasiliensis* trees of the RRIM 600 clone. The latex was fractionated by ultracentrifugation (49,000 *g*, 4 °C, 45 min) to give a floating rubber fraction, known as C-serum (latex cytosol), and a bottom (lutoid) fraction. The bottom fraction was separated off and subjected to further treatment as described below.

3.3. Purification of latex NAD(P)H–QR

B-serum was prepared from 80 g of the bottom fraction by freeze-thawing of the bottom fraction four or five times (at −20 and 37 °C) followed by centrifugation at 10,000 *g* for 20 min (4 °C). The supernatant or B-serum was brought to 30% saturation with acetone solvent under continuous stirring at 4 °C for 10 min. The mixture was centrifuged, and the pellet was discarded. Additional acetone was added to the resulting supernatant to bring it to 50% saturation, and the suspension was stirred for 10 min at 4 °C. The resulting precipitate was collected by centrifugation, freed of residual acetone (by blowing with nitrogen gas), and dissolved in a minimal volume of 50 mM Tris–HCl buffer, pH 8. The solution was heated at 65 °C for 30 min, cooled immediately in an ice bath, and centrifuged at 40,000 *g* for 10 min. The supernatant was concentrated and subjected to further chromatographic purification on a DEAE-Sephacel column (1.7×20 cm) pre-equilibrated with 50 mM Tris–HCl, pH 8. The column was loaded with the heat-treated sample (20 mg) at flow rate of 0.2 ml/min and then washed with the Tris–HCl buffer until the A_{280} of the effluent was zero. The column was next eluted with Tris–HCl buffer containing 0.4 M NaCl, and 3-ml fractions were collected. Fractions containing high NAD(P)H–QR activity were pooled, desalted and concentrated for further purification on a Blue Sepharose column (1.5×5 cm), preequilibrated with 50 mM Tris–HCl, pH 8.0. After washing the column at a flow rate of 0.1 ml/min with Tris–HCl buffer until the A_{280} of the effluent became zero, the bound proteins were eluted with buffer containing a NaCl gradient (0–0.4 M), and 1-ml fractions were collected.

3.4. NAD(P)H–QR activity assay and kinetic analysis

NAD(P)H–QR activity was measured at 25 °C in a Beckman model DU 650i spectrophotometer by following the oxidation of a reduced pyridine nucleotide, as indicated by the decrease in optical density at 340 nm. The disappearance of the absorbance at 340 nm was followed after addition of NAD(P)H (0.2 mM) to an assay mixture containing NAD(P)H–QR in 50 mM Tris–HCl, pH 8, and 0.05 mM menadione (or other quinone substrate, as specified). An extinction coefficient of 6.23 mM^{-1} for NAD(P)H was used for activity calculations. NAD(P)H–QR activity was expressed in katal units, which are equivalent to the conversion of 1 mol of NADH per second. K_m values for pyridine nucleotides were calculated from Lineweaver–Burk plots.

3.5. Molecular weight determination by gel filtration

Molecular weight of the native NAD(P)H–QR was determined by gel filtration on a Sephadex G-200 column (1.5×82 cm) as follows. A 1-ml sample of the DEAE-Sephacel-purified NAD(P)H–QR (5 mg) was loaded on to a Sephadex G-200 column equilibrated with 50 mM Tris–HCl, pH 8. Flow rate was 0.15 ml/min, and 1.0-ml fractions were collected. The molecular weight of NAD(P)H–QR was obtained from a calibration curve of relative log molecular weights of standard markers against their K_{av} values. The following standard markers were used: catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa).

3.6. Effect of pH and temperature

The effects of pH and temperature on the activity of purified NAD(P)H–QR (after Blue Sepharose column) were determined by preincubating samples of NAD(P)H–QR at various pH values from 3 to 10 for 1 h or at temperatures of 10–100 °C for 30 min, or as specified. The mixtures were adjusted back to pH 8 or 4 °C and assayed for NAD(P)H–QR activity. Enzyme activity was determined at pH values of 3–6, 5–8 and 7–10, as obtained with acetate, phosphate or Tris–HCl buffers, respectively. The heat stability of the latex NAD(P)H–QR was determined by incubating enzyme samples at 70, 80 and 90 °C for different times (1–10 h). After incubation, each sample was chilled on ice, and NAD(P)H–QR activity was determined as described in Section 3.4.

3.7. Polyacrylamide gel electrophoresis

SDS–PAGE was performed with 1% SDS and 10% acrylamide, according to method of Laemmli (1970). The standard protein markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14 kDa).

3.8. Protein determination

Protein concentration was determined by method of Lowry et al. (1951), with bovine serum albumin as the standard.

3.9. Correlations between levels of NAD(P)H–QR activity and rubber flow time, dry rubber concentration and flow time, and between flow time and fresh latex and dry rubber per tapping

Forty rubber trees giving high, medium and low levels of rubber yield per tapping were used for the correlation study. The amount of dry rubber, obtained from oven-drying of fresh latex material at 65 °C to constant weight, was used for determinations of dry rubber concentration and yield per tapping. The activity of NAD(P)H–QR was determined in the B-serum and expressed as total activity per tapping. Correlation curves between levels of NAD(P)H–QR activity and rubber flow time, dry rubber concentration and flow time, and between flow time and fresh latex and dry rubber per tapping were constructed.

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