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A NEUTRAL CYTOPLASMIC PHOSPHATASE FROM THE LATEX OF *HEVEA BRASILIENSIS*

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; latex; cytoplasm; acid and neutral phosphatases; metal dependence; rubber biosynthesis.

Abstract—The cytoplasmic serum prepared from the latex of *Hevea brasiliensis* contains an Mg^{2+}-dependent phosphatase named 'neutral cytoplasmic phosphatase' (NCP) which is found at the first tapping of the tree. NCP has been purified and some of its properties have been studied.

INTRODUCTION

Isoprenoid biosynthesis [1] is dependent on the production of acetyl-CoA and ATP following glycolysis [2]. As several phosphoric esters are involved in this metabolic pathway, phosphatases may have an important influence on the production of rubber. Concerning the phosphatases of *Hevea* latex, a non-specific acid phosphatase (EC 3.1.3.2) has been isolated by Jacob and Sontag [3] from the lutoidic serum. A 2′-nucleotidase has been purified by the same authors [4] from the cytoplasmic serum. The presence of a Mg^{2+}-dependent cytoplasmic phosphatase which is not related to cellular organelles has been demonstrated by Ribaillier *et al.* [5]. We report the isolation and some properties of this enzyme.

RESULTS AND DISCUSSION

The phosphatases in the latex of Hevea brasiliensis

Latex is a complex suspension containing mainly rubber particles and several organelles which can be separated by differential centrifugation. Among these organelles, lysosome-like particles (the lutoids) have been shown to contain hydrolytic enzymes and have been extensively studied [6]. After centrifuging the latex (90 min at 45 000*g*), under a cream of rubber, a clear cytoplasmic serum can be collected in which NCP activity is found. Cytoplasmic serum contains, however, an important acid phosphatase activity, which is due to the unavoidable disruption of some of the lutoids localized at the bottom of the centrifuge tubes. Owing to this acid phosphatase activity, which has greater activity than NCP, it was impossible to test specifically for NCP activity, because acid phosphatases could not be totally, or selectively, inhibited by the usual phosphatase inhibitors such as Zn^{2+}, Hg^{2+}, Cu^{2+}, Pb^{2+}, molybdate, phosphate, pyrophosphate, fluoride or arsenate. Attempts to separate the two activities in the first steps of purification were unsuccessful.

Characterization of NCP activity—purification

NCP may be distinguished from acid phosphatase by its electrophoretic mobility, its strong activation by Mg^{2+}, and by its higher activity at pH 6.8 rather than pH 5.6, which is the pH optimum of the acid phosphatase [3]. The different steps of purification of the enzyme were therefore followed by pH activity profiles and polyacrylamide gel electrophoresis. The principle of the experiments was to start from a cytoplasmic serum with predominant acid phosphatase activity and an optimal pH of 5.6, to the step where the pH optimum was 6.8 (optimal pH of NCP) and polyacrylamide gel electrophoresis showed only one band of phosphatase activity. The purification of NCP (Fig. 1) was achieved as indicated in Experimantal.

Properties

The reported properties of NCP were studied with the enzyme preparation from step 4 (see Experimental). NCP hydrolyses *p*-nitrophenylphosphate (*p*NPP) with an optimal pH at 6.8 and half maximal activity is observed at pH 5.0 and 8.8. The nature and molarity of the buffer have little effect on activity. Such an optimal pH is close to the physiological pH of the cytoplasmic serum (6.7 ± 0.2) and the latex (6.9 ± 0.2). The specific activity of the purified enzyme was 0.6 μmol *p*NPP hydrolysed/min/mg protein. The enzyme has a pI of 4.77 ± 0.02 and is therefore anionic at physiological conditions like most of the proteins of the cytoplasmic serum [7].

After dialysis against 0.1 M Tris–maleate buffer at pH 6.8 containing 2 mM mercaptoethanol (MSH), the NCP shows a small residual activity which may be due

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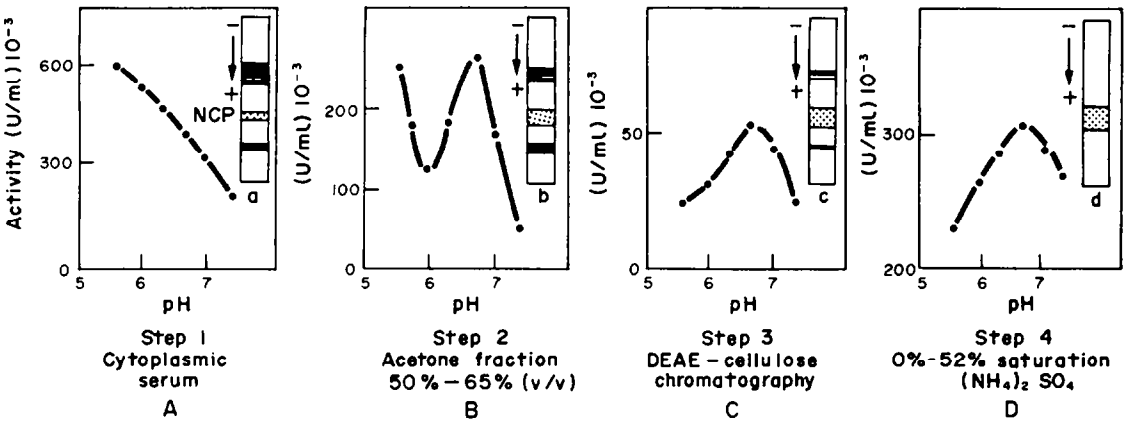

Fig. 1. pH activity curves (A, B, C, D) and polyacrylamide gel electrophoresis (a, b, c, d) during purification of NCP. The gels were stained for both acid (■) and neutral (▨) phosphatase activities as described under Experimental.

to an incomplete elimination of cations, or to reagent contamination by traces of activating metal ions. The optimal pH is also independent of Mg^{2+} concentration suggesting that NCP activation by Mg^{2+} is independent of pH between 6.4 and 7.1.

Maximal activity with Mg^{2+} is shown with 10 mM Mg^{2+} which corresponds to physiological values (results not shown). With saturating concentrations of pNPP (5 mM), the curve of activation by Mg^{2+} has a hyperbolic shape, suggesting that Mg^{2+} acts both on the activity of NCP and on its affinity for the substrate. In the presence of 10 mM EDTA at pH 6.8, the enzyme is completely inactivated but the addition of Mg^{2+} restores initial activity. These results confirm that NCP is Mg^{2+}-dependent. However the enzyme may be activated by other metal ions, as shown below.

In contrast with acid phosphatase (EC 3.1.3.2), Table 1 shows that NCP specifically hydrolyses phosphate esters with an aromatic residue, particularly α- or β-naphthyl-phosphates (NAP) and phenylphosphate. The presence of aromatic compounds such as phenylalanine (0–10 mM) or tyrosine (0–1 mM) in the assays, had no effect on the enzymatic activity, even when NCP was preincubated in the presence of these analogues (30 and 5 mM, respectively). It is of interest that some phosphate esters of sugar metabolism and some nucleotides are not hydrolysed by NCP. The enzyme is also inactive with the phosphate diester, bis *p*-nitrophenylphosphate. These results show that the substrate of the enzyme must have both a phosphate monoester bond and an aromatic radical; in addition this specificity probably indicates that NCP could not hydrolyse the phosphate ester intermediates of isoprenoid biosynthesis. So far, we have found the enzyme only in the latex of *Hevea brasiliensis*, even in trees which had never been previously tapped. The cytoplasmic sera of other laticiferous plants such as *Artocarpus communis*, *Ficus elasticodes*, *Plumaria rubra*, *Fontumia africana*, *Euphorbia unispina* and *Tabernae montana-crassa* did not show the presence of any NCP-like enzymes by electrophoresis.

A K_m value of 0.4 mM was found for *p*NPP at 35°. For other substrates, the K_m values were determined by following *p*NPP hydrolysis in the presence of these substrates acting as competitive inhibitors. Under these conditions, the K_i value corresponds to the K_m value of the second substrate. The K_m values were 0.4 mM for α-NAP, 0.7 mM for phenylphosphate, 0.3 mM for phenolphthalein-phosphate and 0.5 mM for phenolphthalein-diphosphate.

The apparent MW of NCP was 65 000 ± 5 000 [8]. Monovalent ions such as K^+, NH_4^+ or Li^+, as their chlorides, had no effect on the NCP at a concentration

Table 1. Substrate specificity of NCP

Substrate	Relative activity (%)
Substrates with an aromatic group	
p-Nitrophenyl phosphate	100
α-Naphthyl phosphate	60
β-Naphthyl phosphate	49
Phenylphosphate	50
Phenolphthalein-phosphate	24
Phenolphthalein-diphosphate	14
Bis *p*-nitrophenyl phosphate	4
Phosphoric esters of sugar metabolism	
Fructose-6-phosphate	4
Fructose-1,6-diphosphate	4
Glucose-6-phosphate	6
Erythrose-4-phosphate	18
2,3-Diphosphoglyceric acid	5
Ribose-5-phosphate	1
2-Phosphoglycerate	0
Galactose-1-phosphate	0
α-Glycerophosphate	0
Nucleotides	
NADP	4
NADPH	6
Cytidine-triphosphate	1
Uridine-diphosphoglucose	0
Adenosine-5'-triphosphate	11
Adenosine-5'-diphosphate	9
Adenosine-5'-monophosphate	4
Uridine-5'-monophosphate	7
Guanosine-5'-triphosphate	8
Coenzyme A	3

Incubations were performed with 0.5 ml of 3 mM substrates in 0.1 M Tris–maleate buffer (pH 6.8) and 10 mM $MgCl_2$ at 30°. The reaction was started by adding 10 μl of enzyme, stopped after 15 min with 1 ml of 5% ammonium molybdate in 5 N H_2SO_4, and tested for released Pi [12].

Table 2. Effect of different cations

Metal ion	Relative activity (%)
No addition	100
K^+	100
NH_4^+	98
Li^+	98
Hg^{2+}	0
Zn^{2+}	27
Cu^{2+}	53
Sr^{2+}	45
Ba^{2+}	73
Ca^{2+}	84
Co^{2+}	413
Mg^{2+}	394
Mn^{2+}	265
Ni^{2+}	198

Incubations were performed at pH 6.8 in Tris–maleate buffer, with 5 mM *p*NPP. Metal ions were used at a concentration of 10 mM except for Hg^{2+}, Cu^{2+} and Zn^{2+} (1 mM).

of 10 mM. The enzyme was strongly inhibited by Hg^{2+} and Zn^{2+} and to a lesser extent by Sr^{2+}, Cu^{2+}, Ba^{2+} and Ca^{2+} (Table 2). In addition to Mg^{2+}, the enzyme was also activated by Co^{2+}, Mn^{2+} and Ni^{2+}. Maximal activation by these metal ions occurred at *ca* 5 mM. No synergistic effects were noted between these different activators.

pH stability was studied at 0°, between pH 3.4 and 8.5 (0.1 M acetate or Tris–maleate buffers), in the presence and absence of 10 mM Mg^{2+} for 60 min. The enzyme was stable between pH 5.5 and 6.5 and still showed 95% activity at pH 8.5 and 60% activity at pH 3.6. Inactivation at acidic pH was partially protected by Mg^{2+}. The enzyme was stable for at least 45 min at 45° in 0.1 M Tris–maleate buffer, pH 6.8. After 10 min at 65°, the residual activity was 50% and the enzyme was completely inactivated after 9 min at 70°. The inactivation reaction showed first-order kinetics. When stored for 5 days at 0° in 0.1 M Tris–maleate buffer at pH 6.8, the enzyme lost about 30% of its initial activity. The same result was observed in the presence of 10 mM Mg^{2+} or 10 mg/ml bovine serum albumin. After 3 months at −15°, the enzyme showed 70% of its initial activity, and could be completely reactivated by adding MSH at 2 mM.

At pH 6.8, the activation energy was 15 000 cal/mol. The Q_{10} was 2.30 between 25 and 35°, 2.15 between 35 and 45°. The enzyme was inhibited 50% by pyrophosphate (15.5 mM), fluoride (9 mM), arsenate (6.5 mM), phosphate (4.5 mM), molybdate (0.4 mM) and *p*-chloromercuribenzoate (18 μM). Citrate showed little inhibition.

EXPERIMENTAL

Latex was collected at 7 am according to ref. [7] from 20 6-yr-old rubber trees which had been regularly exploited since the age of 4 yr. The pooled latex (3 l.) was kept in ice with 1 M triethanolamine (TEA)–HCl buffer (pH 7), 0.3 M mannitol and 10 mM MSH (6 ml/100 ml latex). The suspension was centrifuged at 4°, for 10 min at 4000*g*; the lutoid fraction at the bottom and some coagulated rubber at the top were discarded. The middle fraction, called the 'white fraction', was centrifuged at 45 000*g* for 90 min at 4° to yield *ca* 800 ml of cytoplasmic serum which was lyophilized. Pooled lyophilisates were used for NCP purification.

Phosphatases assays. Unless otherwise stated, the enzyme (10–50 μl) was assayed with 1 ml of 5 mM *p*NPP containing 10 mM Mg^{2+} and 0.1 M Tris–maleate buffer, pH 6.8. After 5 min preincubation at 35°, the reaction was started by adding the enzyme and stopped after 20 min with 0.5 ml 1 M KOH containing 0.1 M EDTA. $A_{400\,nm}$ of *p*NP was read according to [10]. One enzyme unit is the amount which catalyses the hydrolysis of 1 μmol of *p*NPP per min under the experimental conditions. Inorganic phosphate was measured by the method of [11]. Magnesium concns were determined by complexometry using EDTA and eriochrom-T black according to [12]. Chlorides for NaCl gradients were tested according to [13]. Protein concns were measured by the method of [14], after extraction performed according to [15]. $(NH_4)_2SO_4$ concns were calculated by reference to the diagram of [16]. Fibrous DEAE-cellulose (Pfaltz and Bauer) was precycled as described [17]. The column was 50 × 4.5 cm and contained *ca* 500 ml of swollen ion exchanger. Protein elution was recorded at 280 nm. Electrofocusing expts were performed [18] with ampholines, pH 3.5–10 and pH 4–6, in a sucrose gradient and a 110 ml column. For MW determinations, a Pharmacia column K 25/100 of Sephadex G 200 was prepared and equilibrated with 0.05 M TEA–HCl buffer at pH 6.8, containing 0.5 M NaCl [19]. The proteins used for calibration were horse heart cytochrome *c* (12 400), bovine ribonuclease (13 700), bovine serum albumin (monomer: 67 000, dimer: 134 000), pig heart malate dehydrogenase (70 000), rabbit muscle aldolase (154 000). The void vol. was determined with Blue Dextran 2000 (Pharmacia, Uppsala). Electrophoresis using 7.5% polyacrylamide gels (9.0 × 0.8 cm) was carried out in a Shandon apparatus [20], the gels being stained for phosphatase activities according to [21]. They were successively incubated at pH 5 for acid phosphatases then at pH 6.5 in the presence of Mg^{2+} for NCP activity. Proteinograms were stained with 0.1% naphthol blue B standard in 0.7% HOAc for 30 min, and destained electrophoretically at 7 mA/tube, in 2% HOAc. Dialyses were performed with cellulose nitrate ultra thimbles for small vols or with cellulose tubular bags for large vols.

Purification. Step 1: 30 g of lyophilized cytoplasmic serum were dissolved at 0° with H_2O containing 2 mM MSH. An insoluble pellet was discarded after centrifugation (45 000*g* for 90 min) and the pH of the supernatant was adjusted to 7 with 1 M TEA–HCl buffer pH 7. This fraction corresponds to 800 ml of fresh latex and contains *ca* 10 g of proteins. At this stage, the pH optimum for phosphatase activity is 5.6 and indicates the predominance of the acid phosphatase activity in the serum which is shown by polyacrylamide gel electrophoresis (Fig. 1A,1a). *Step 2:* The supernatant serum was submitted to an Me_2CO fractionation; the fraction which precipitated between 50 and 65% (v/v) Me_2CO was dissolved in 100 ml 0.1 M TEA–HCl buffer, pH 7. At this stage, the pH optimum profile (Fig. 1B) showed two peaks of phosphatase activity: one at pH 5.6 corresponding to acid phosphatase and the other at pH 6.8, corresponding to NCP. Polyacrylamide gel electrophoresis still showed the presence of acid phosphatase activity (Fig. 1b). *Step 3:* The soln was chromatographed through DEAE-cellulose. About two-thirds of the remaining acid phosphatase was removed by

washing the column with the equilibration buffer. NCP was eluted by applying a linear gradient of buffered NaCl (0–0.3 M) at a chloride concn of 0.18 M. The active fractions were pooled but still showed a small acid phosphatase activity by polyacrylamide gel electrophoresis (Fig. 1c). The pH optimum of the pooled fractions was 6.8, indicating that NCP was the main phosphatase activity in this fraction (Fig. 1C). *Step 4:* The soln was concd by dialysis against solid sucrose and brought to 52% saturation with $(NH_4)_2SO_4$. The pellet, which contained NCP, was dissolved in 0.1 M TEA–HCl buffer at pH 6.8. The pH activity profile showed a single maximum at pH 6.8 (Fig. 1D). After polyacrylamide gel electrophoresis, the gels showed one band of phosphatase which corresponded to NCP, and one major band of protein of the same mobility (Fig. 1d). The isolation of NCP was confirmed by isoelectric focusing which showed a single phosphatase activity peak. The enzyme prepn was stored at −30° for up to 3 months without significant loss of activity.

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