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PURIFICATION OF ACID PHOSPHATASE I FROM GERMINATING SEEDS OF *VIGNA SINENSIS*

TAPAN K. BISWAS, MICHELE PROMO* and BANI BISWAS

Department of Pathology, University of Chicago, Chicago, IL 60637, U.S.A.

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Key Word Index—*Vigna sinensis*; Fabaceae; bean; germination; purification; acid phosphatase.

Abstract—Acid phosphatase I (AP-I) is the major isoform of *Vigna* acid phosphatase. It is constitutively expressed in seed cotyledons during germination. AP-I was separated from other isoforms and purified to homogeneity by three simple purification steps; $(NH_4)_2SO_4$ precipitation, and phosphocellulose and DEAE-cellulose column chromatography. The activity of AP-I was not affected by 1 mM Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} or Pb^{2+}, but severely inhibited by 1 mM Cu^{2+}, Fe^{3+}, Hg^{2+}, Mo^{6+} or Zn^{2+}. AP-I has both phosphatase and pyrophosphatase activities, and is highly stable even at 50°.

INTRODUCTION

The cotyledons of leguminous plants mainly contain food materials which are supplied to the embryo during seed germination. The activity of hydrolytic enzymes as well as the metabolic function of seed cotyledons increase with germination to release the storage food materials for the growing embryo [1, 2]. These enzyme activities in cotyledons are enhanced by the attachment of embryo, suggesting that there is some communication between the cotyledons and the embryos to regulate hydrolysis of storage materials according their requirement [2–4].

Phosphate, an important molecule for cellular growth, is involved in many different biological reactions [5]. The liberation of phosphate from phosphate-ester is mainly effected by phosphatase, an enzyme widely found in nature [6–8]. Four different isoforms of acid phosphatase are present in *Vigna sinensis* [9, 10]. Two of these (AP-I and AP-II) are constitutively expressed and another two (AP-III and AP-IV) are developmentally regulated. AP-I has both phosphatase and pyrophosphatase activities and is always present in germinating seeds. Since AP-I is the major phosphatase in germinating seeds of *V. sinensis*, we developed a simple purification procedure to separate AP-I from other isoforms and to purify it to homogeneity. In this communication, the purification procedure and some properties of AP-I have been described.

RESULTS AND DISCUSSION

The main objective of this study was to purify the major acid phosphatase from *V. sinensis*. Details are given in the Experimental section. Since AP-I is the only isoform of *Vigna* acid phosphatase that binds to a phosphocellulose column, AP-I was separated from other isoforms of acid phosphatase (AP-II, AP-III and AP-IV) by phosphocellulose column chromatography. The specific binding of AP-I to phosphocellulose suggests that AP-I has higher affinity for phosphate-ester compounds compared with other isoforms. AP-I was further purified to homogeneity by DEAE-cellulose chromatography. The purity of AP-I is judged by polyacrylamide gel electrophoresis followed by activity staining or by protein staining with Coomassie Blue. The final preparation was purified over 200-fold and showed one protein band in polyacrylamide gel electrophoresis.

The M_r of AP-1 was determined by Sephadex G-150 gel filtration to be 150 k. The activity of AP-I was inhibited by 80% in the presence of 1 mM of one of the five metal ions Cu^{2+}, Fe^{3+}, Hg^{2+}, Mo^{7+} or Zn^{2+} whereas 1 mM of Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} or Pb^{2+} did not change AP-I activity. Substrate-specificity analysis showed that AP-I used *p*-nitrophenyl phosphate (*p*NPP) and pyrophosphate most efficiently and equally well as substrate among various phosphate-ester compounds, as did *Aspergillus* acid phosphatase I [11]. This result suggests that AP-I might be a multi-functional enzyme, which carries both phosphatase and pyrophosphatase activities.

To investigate further whether the phosphatase and pyrophosphatase activities were associated with a single protein, pH and temperature optima, and heat inactivation of both activities were determined. The pH optimum

*Present address: Department of Chemistry, University of Minnesota, Minneapolis, MN55455 U.S.A.

was determined between pH 4.0 and 9.0. The highest activities of both phosphatase and pyrophosphatase were found at pH 5.0 like other acid phosphatases isolated from wheat roots [12] and pea [13]. At pH 5.0, the optimum temperature of both phosphatase and pyrophosphatase activities was 55°. However, at a higher temperature both activities declined rapidly. To determine the thermal stability at pH 5.0, the enzyme was preincubated at 60° or 65° for different times and then *p*NPP or pyrophosphate was added as substrate to start the reaction. Apparently, there were two forms of enzyme activity: heat-stable and heat-labile forms. After 45 min of preincubation at either of the two temperatures, 50% of enzyme activities were retained. The overall patterns of heat inactivation of acid phosphatase and pyrophosphatase were very similar. The similar behaviour of both activities in the AP-I fraction suggests that these phosphatase and pyrophosphatase activities were associated with a single protein or with a very similar type of protein.

EXPERIMENTAL

Materials: *p*NPP, α-naphthyl phosphate, Fast Blue RR salt, Triton X-100 and DEAE-cellulose were from Sigma. Phosphocellulose was from Whatman.

Enzyme assay. Acid phosphatase activity was measured by incubating enzyme with 0.1 mM *p*NPP in acetate buffer (pH 5) at 50° for 20 min as described in ref. [9]. The reaction was stopped by addition of 0.1 M NaOH and the A_{410} nm developed from the liberated *p*-nitrophenol (*p*NP) was measured. Pyrophosphatase activity was measured under similar reaction conditions except pyrophosphate was used as a substrate and the liberated inorganic phosphate was measured according to the method of ref. [14].

Protein concentration. This was determined colorometrically according to the method of ref. [15] or by *A* at 280 nm.

Polyacrylamide gel electrophoresis (PAGE). PAGE of phosphatase on a 7% gel was carried out followed by phosphatase activity staining [9] or protein staining with Coomassie Brilliant Blue R [16].

Enzyme purification: Seeds of *Vigna sinensis* were immersed in 0.2 mg ml^{-1} $HgCl_2$ soln for 10 min (for surface sterilization) and then washed several times with dist. H_2O. Seeds were germinated in the dark at 28°. The cotyledons of 48 hr germination seeds were sepd from the embryos and a 10% homogenate was prepd in buffer A (10 mM Tris–HCl, pH 7.4) containing 0.1% Triton X-100. The homogenate was strained through cheesecloth and then centrifuged at 10 000 *g* for 20 min. The supernatant was collected and protein was pptd by 40–60% $(NH_4)_2SO_4$ satn. The pptd protein was collected by centrifugation and dissolved in buffer A. After dialysis against buffer A, 170 mg protein of 40–60% $(NH_4)_2SO_4$ fr. was loaded on to a phosphocellulose column (2.4 × 20 cm) and then washed with 400 ml buffer A. The phosphocellulose-bound protein was eluted with a linear gradient of 0–300 mM KCl. The 4 ml frs were collected and phosphatase activity was measured. The PAGE sepn patterns of the eluted acid phosphatase in the active frs were visualized by activity staining. AP-I, but not other acid phosphatase isoforms (AP-II, AP-III and AP-IV), were bound to phosphocellulose.

The active frs from the phosphocellulose column were pooled and dialysed against buffer A containing 50 mM KCl. The dialysed pooled fr. was loaded on to a DEAE-cellulose column (1.2 × 20 cm) and then washed with buffer A containing 50 mM KCl. The bound protein was eluted with a 0.05–0.3 M KCl gradient and 4-ml frs were collected. After measuring phosphatase activity, the active frs were pooled, dialysed against buffer A and then stored at − 20°.

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