Phosphatases are enzymes usually classified as acid or alkaline depending on their pH optimum for catalytic activity being below or above pH 7. Acid phosphatases (APs; EC 3.1.3.2) are ubiquitous enzymes ranging from microorganisms (fungi and bacteria) to plants and animals [1, 2]. APs catalyze the hydrolysis of phosphate monoesters, and their function seems to be the release, transport, and recycling of Pi, a crucial macronutrient for cellular metabolism and bioenergetics. Plant APs have been isolated and characterized from several sources [2], and some of these have been crystallized [3, 4]. These enzymes, homodimeric or heterodimeric glycoproteins, can be intracellular (localized in cytoplasm and vacuole) or extracellular (localized in cell wall) [5].

Purple acid phosphatases (PAPs) represent a distinct class of acid phosphatases. Their typical absorbance spectrum with maximum at 530-560 nm, with a characteristic purple color, is due to a charge-transfer transition between a tyrosine residue and a coordinated ferric ion [4]. These enzymes contain a Fe(III)-Me(II) metal center, where Me(II) can be Zn, Mn, or Fe ([6, 7] and references therein). Purple acid phosphatases from bacteria, mammals, and plants contain seven highly conserved metal ligand residues (DXXG-GXXDXXY-GNH[(D/E)-VXXH-GHXH]; bold letters indicate metal ligand residues, dashes indicate separation between blocks) forming dimetallic active centers. Moreover, the structure of the catalytic site and other domains of purple acid phosphatase are also highly conserved [8]. Plants contain two major groups of PAPs: small PAPs, 35-40 kDa monomeric proteins homologous to mammalian enzymes, and large PAPs, 110-130 kDa homodimeric proteins, with or without a disulfide bridge between the two subunits [9].

A large number of plant species exude a milky, variously colored sap known as latex. Contained in laticifers, specialized cells forming vessel-like structures, plant latex is a complex environment with a diversified composition that includes terpenoid compounds, alkaloids, and a number of proteins. Several proteins had been well characterized in the latex of the Mediterranean shrub Euphorbia characias and, very recently, a soluble metallo-

**Purification, Primary Structure, and Properties of Euphorbia characias Latex Purple Acid Phosphatase**

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**Abstract**—A purple acid phosphatase was purified to homogeneity from Euphorbia characias latex. The native protein has a molecular mass of 130 ± 10 kDa and is formed by two apparently identical subunits, each containing one Fe(III) and one Zn(II) ion. The two subunits are connected by a disulfide bridge. The enzyme has an absorbance maximum at 540 nm, conferring a characteristic purple color due to a charge-transfer transition caused by a tyrosine residue (Tyr172) coordinated to the ferric ion. The cDNA nucleotide sequence contains an open reading frame of 1392 bp, and the deduced sequence of 463 amino acids shares a very high degree of identity (92-99%) to other purple acid phosphatases isolated from several higher plants. The enzyme hydrolyzes well p-nitrophenyl phosphate, a typical artificial substrate, and a broad range of natural phosphorylated substrates, such as ATP, ADP, glucose-6-phosphate, and phosphoenolpyruvate. The enzyme displays a pH optimum of 5.75 and is inhibited by molybdate, vanadate, and Zn²⁺, which are typical acid phosphatase inhibitors.

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Phosphatases are enzymes usually classified as acid or alkaline depending on their pH optimum for catalytic activity being below or above pH 7. Acid phosphatases (APs; EC 3.1.3.2) are ubiquitous enzymes ranging from microorganisms (fungi and bacteria) to plants and animals [1, 2]. APs catalyze the hydrolysis of phosphate monoesters, and their function seems to be the release, transport, and recycling of Pi, a crucial macronutrient for cellular metabolism and bioenergetics. Plant APs have been isolated and characterized from several sources [2], and some of these have been crystallized [3, 4]. These enzymes, homodimeric or heterodimeric glycoproteins, can be intracellular (localized in cytoplasm and vacuole) or extracellular (localized in cell wall) [5].

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A large number of plant species exude a milky, variously colored sap known as latex. Contained in laticifers, specialized cells forming vessel-like structures, plant latex is a complex environment with a diversified composition that includes terpenoid compounds, alkaloids, and a number of proteins. Several proteins had been well characterized in the latex of the Mediterranean shrub Euphorbia characias and, very recently, a soluble metallo-
protein phosphatase/phosphodiesterase, containing one Ca(II) and one Mg(II) ion, was purified to homogeneity from it ([10] and references therein).

In this paper we describe the purification and some biochemical characteristics of a purple acid phosphatase (ELPAP) extracted from E. characias latex. The substrate specificity, effect of inhibitors, activation energy, pH effect, and other biochemical characteristics of the enzyme have been investigated. Moreover, we isolated and sequenced the cDNA encoding ELPAP, and here we report the nucleotide and the deduced amino acid sequences showing the conserved residues involved in several domains. This study leads us to learn more about the biochemistry of the proteins of Euphorbia latex and to hypothesize a possible physiological role of the joined action of two secreted metalloproteins.

MATERIALS AND METHODS

ATP, ADP, AMP, GMP, 4-nitrophenyl phosphate (p-NPP), glucose-1-phosphate, glucose-6-phosphate, inositol-1-phosphate, phosphoenolpyruvate, pyridoxal-5-phosphate, 2,3-bisphosphoglycerate, iron chloride, calcium chloride, magnesium chloride, manganese chloride, copper chloride, zinc chloride, nickel chloride, cobalt chloride, cadmium chloride, sodium fluoride, sodium molybdate, and sodium vanadate, tartrate, citrate, and EDTA were from Sigma Chemical Co (USA).

Euphorbia characias latex, drawn from cut branches, was collected at several locations in southern Sardinia (Italy), immediately frozen at −80°C, lyophilized, and stored at −20°C until use.

Enzyme purification. ELPAP was purified from the lyophilized E. characias latex by the following steps at 4°C unless otherwise indicated.

Step 1. Acetone powder. The lyophilized material was poured into 1 liter of cold acetone and kept for 45 min with constant stirring at −20°C. The acetone powder was collected by filtration on a Buchner funnel. Acetone powder (30 g) was mixed with 700 ml H2O with continuous stirring for 45 min. The acetone powder was poured into 1 liter of cold acetone and kept for 45 min unless otherwise indicated.

Step 2. Ammonium sulfate fractionation. The supernatant was made 25% saturated with ammonium sulfate with constant stirring for 30 min and centrifuged at 14,300 × g for 30 min. The precipitate was discarded, and the supernatant was brought to 80% saturation with ammonium sulfate with constant stirring for 30 min and centrifuged at 14,300 × g for 30 min. The pellet was dissolved in 40 ml of 10 mM Tris-HCl buffer, pH 7.0, and dialyzed for 12 h against the same buffer.

Step 3. DEAE-cellulose chromatography. The dialyzed protein was loaded onto a DEAE-cellulose column (2.8 × 14 cm) equilibrated and washed with 10 mM Tris-HCl buffer, pH 7.0. The enzyme was then eluted with 100 mM Tris-HCl buffer, pH 7.0. The fractions with the same specific activity were pooled and concentrated by ultrafiltration.

Step 4. Gel-filtration chromatography. The concentrated solution was loaded onto a column (2 × 100 cm) of Sephacryl S-200 (fine grade) equilibrated and eluted at 4°C with 100 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl. The fractions with the highest specific activity were pooled and concentrated by ultrafiltration.

Analytical polyacrylamide gel electrophoresis (PAGE). Electrophoresis in nondenaturing conditions was performed as previously described [11]. The protein band with ELPAP activity was detected after the electrophoretic run by staining the gel in 100 mM Na-acetate buffer, pH 5.75, containing 1 mM p-NPP.

SDS-PAGE was carried out according to Weber and Osborne [12]. The protein samples for SDS-PAGE were heated at 100°C for 5 min in 10 mM Tris-HCl buffer, pH 7.0, containing 1% SDS in the presence and in the absence of 100 mM 2-mercaptoethanol. For molecular mass determination, the standards used were: myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and α-lactalbumin (14.2 kDa).

Deglycosylation analysis. Protein samples were denatured by boiling for 5 min and then deglycosylated with N-glycosidase (5 mg/ml) in 50 mM Na-Pi buffer, pH 7.2, at 37°C for 12 h. Controls were performed in samples without N-glycosidase. Protein samples were analyzed by SDS-PAGE.

Gel filtration and molecular mass determination. The Mr under nondenaturing conditions was estimated by gel filtration using a column (2 × 100 cm) of Sephacryl S-200 (fine grade) equilibrated and eluted at 4°C with 100 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl. The distribution coefficient Kd was obtained as described [13] using blue dextran to measure the void volume V0 and tyrosine to measure the total volume VT. The standards used were: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (31 kDa), and cytochrome c (12.4 kDa).

Metal content. The amounts of Fe3+, Mn2+, and Zn2+ were measured by atomic absorption using an IRIS Intrepid Spectrometer (Thermo Elemental, USA). The spectral lines chosen were 248.300 nm for Fe3+, 259.373 nm for Mn2+, and 206.200 nm for Zn2+.

Protein determination. Protein concentration was measured by the Bradford method [14] using bovine serum albumin as a standard for the calibration curve.

Spectrophotometric features. Spectrophotometric determinations were recorded with an Ultrospec 2100 spectrophotometer (Biochrom Ltd, UK).

Enzyme assays using p-NPP as substrate. For routine measurements of ELPAP activity, the hydrolysis of p-NPP was determined by direct spectrophotometric meas-