Chemistry of Natural Compounds, Vol. 40, No. 5, 2004

PHYSICOCHEMICAL PROPERTIES OF COTTON-LEAF PEROXIDASE

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UDC 577.124.5:633.511

The physicochemical properties of peroxidase isolated from cotton leaves were investigated. The optimal pH value for exhibiting activity was 4.7; temperature, 30°C. The Michaelis constant was 2.3 mM. Cotton-leaf peroxidase has a very high affinity for benzidine.

Key words: peroxidase, cotton, thermal stability, substrates.

Peroxidase is one of the most widely distributed plant enzymes and has been studied in horseradish, wheat, alfalfa, peas, barley, potatoes, etc. [1].

All plant organs contain peroxidase. This is indicative of its universal functions. Therefore, we isolated peroxidase from various cotton organs: stem, root, pods, integument, fiber, and aging leaves in various vegetative stages. Peroxidase isolated from cotton leaves during extensive opening of the pods was most active.

The specific activity of peroxidase (U/mg) isolated from various cotton organs was 9.1 from stem; 8.9, root; 7.8, pods; 3.1, integument; 2.05, fiber; and 120, leaves.

Gel filtration over TSK-HW-65 gel produced three fractions, of which the second fraction had peroxidase activity. Further purification by ion-exchange chromatography over DEAE-cellulose gave a purified peroxidase activity of 840 U/mg (Fig. 1). This fraction was used in the investigations.

It is important in the study of enzymes to consider the optimal conditions needed for reactions to occur. These include thermal stability, substrate specificity, isoelectric points, and optimum pH.

Proteins with peroxidase activity at pH 3.5-4.7 were determined by isoelectric focusing on PAAG-plates containing ampholines in the pH range 3.5-9.5 (Fig. 2).

Plant isoperoxidases consist of acidic and basic enzyme forms. The cotton-leaf peroxidase that we obtained was mainly the acid forms. By reviewing the literature data and ours [2, 3], it can be seen that acidic isoperoxidases have a higher specific activity for benzidine, *o*-phenylendiamine, luminol, ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and diaminobenzidine whereas basic ones are more specific for guaiacol, phenyl-antipyrine, and pyrogallol. Cotton-leaf peroxidase has the highest affinity for benzidine, *o*-dianisidine (ODN), and ABTS (Table 1).

The enzyme activity as a function of pH is a superposition of two pH dependencies. These are intrinsic to the substrate conversion and conformational changes of the enzyme globule. Standard conditions for measuring the peroxidase activity are known. A more appropriate characteristic of isoperoxidase is the pH of optimum stability, which is independent of the substrate, is determined by the nature of the enzyme, and, as a rule, is close to the p_i of the protein.

Data have been published [2] indicating that the enzyme has different optimal pH values depending on the source and is active at pH values from 4 to 8. It is also known that peroxidase isoforms from a single plant are active at different pH values [1]. Local pH changes in the cell probably regulate the inclusion of some isoenzymes or others in the oxidation of phenolic compounds. This justifies the existence of multiple peroxidase forms.

Various buffers at pH 3.6-9 were used to determine the optimum pH values for oxidation by peroxidase isolated from cotton leaves. Figure 3a shows peroxidase activity as a function of pH using benzidine as substrate. The optimum pH value was 4.7.

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 0009-3130/04/4005-0506

TABLE 1. Specificity of Cotton-Leaf Peroxidase (units/mg protein)

Substrate	Wavelength, nm	Specific activity, U/mg
o-Phenyldianisidine	460	99
o-Dianisidine	460	776
Benzidine	620	840
ABTS	405	820

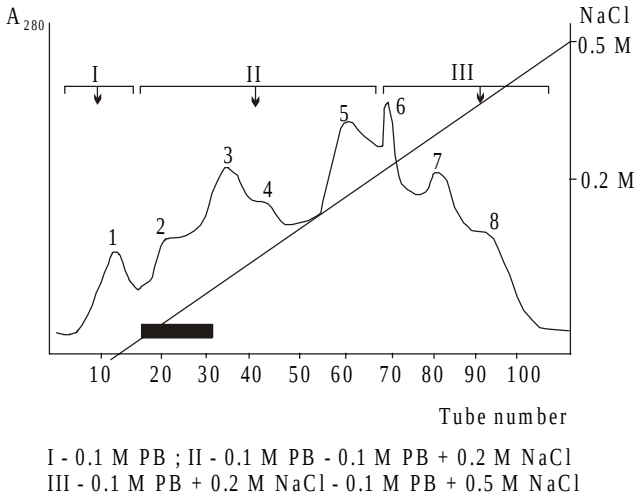

Fig. 1

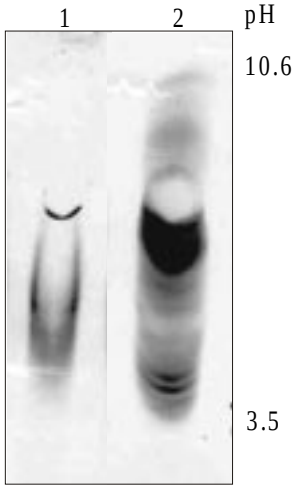

Fig. 2

Fig. 1. Ion-exchange chromatography over TSK-DEAE-cellulose (5.0 × 2.5 cm) of cotton-leaf peroxidase (second fraction eluted over TSK-HW-65) by phosphate buffer (PB, 0.1 M), pH 6.0. Unbound fraction (1), acidic fraction (2-7).
Fig. 2. Isoelectric focusing of purified cotton-leaf peroxidase after ion-exchange chromatography over TSK-DEAE-cellulose. Purified fraction (1), pH 3.5-10.65 controls (2).

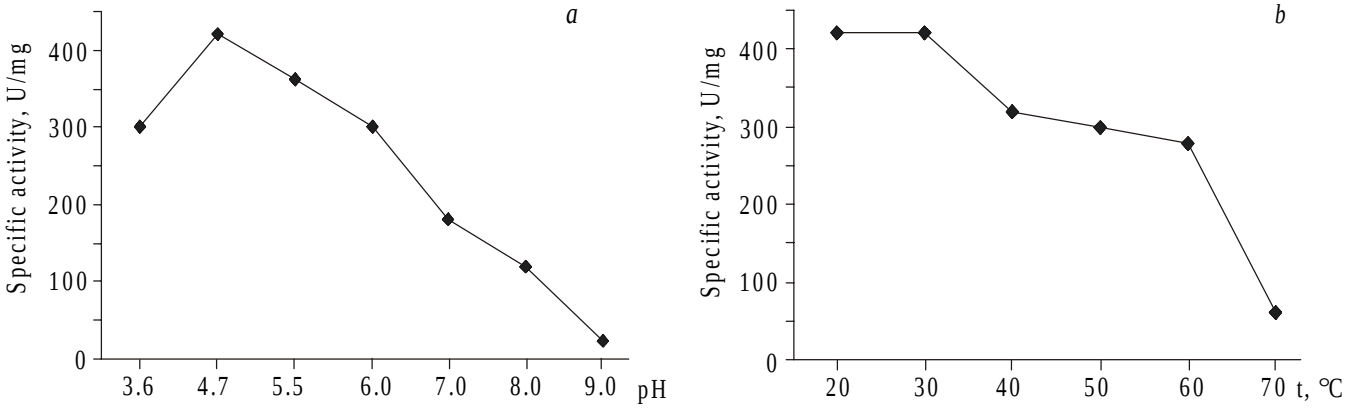

Fig. 3. Specific activity of peroxidase at various pH values (a) and temperatures (b).

Horseradish-root peroxidase is the enzyme with which, as a rule, the properties of peroxidases from cell cultures with high thermal stability are compared. This property is also observed for preparations of peroxidase isolated from liquid culture of cotton [4] and cowpea [5] cells. Cotton peroxidase remained fully active up to 60°C. The cotton-leaf peroxidase activity as a function of temperature is plotted in Fig. 3b. The optimum temperature is 30°C. As the temperature increases, the enzyme activity decreases: at 40-60°C, to 76 and 66%; at 70°C, only 14% activity remains.

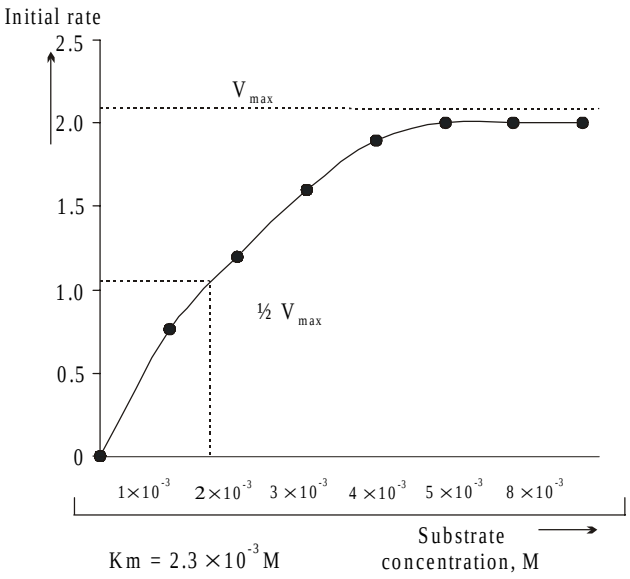

Fig. 4. Michaelis—Menten constant of cotton-leaf peroxidase.

The Michaelis constant (K_m) and rate (K_{cat}) for benzidine hydrolysis were determined to characterize the catalytic activity of purified cotton-leaf peroxidase. The kinetics of catalytic decomposition of benzidine by peroxidase were studied at pH 4.7.

The Michaelis constant was found at constant peroxidase and H_2O_2 concentrations from the change of benzidine concentration.

Figure 4 shows K_m, 2.3 mM, calculated at 20°C at various benzidine concentrations.

Thus, the physicochemical properties of peroxidase isolated from cotton leaves were determined.

EXPERIMENTAL

Aging cotton leaves (*Gossypium hirsutum*) were used in the investigations. Cell walls were destroyed by liquid N_2 with subsequent extraction by phosphate buffer (PB, 0.1 M) at pH 6.0. Peroxidase was precipitated with ammonium sulfate and purified by gel filtration over TSK-HW-65 gel followed by ion-exchange chromatography over DEAE-cellulose using the same buffer.

Peroxidase activity was determined spectrophotometrically by the Boyarkin method [6].

The specific activity was calculated using the formula:

$$d = \Delta E \times 2/a \cdot b,$$

where d is the specific activity, ΔE is the absorption change during 1 min at the corresponding wavelength, a is the amount of protein in mg/mL, and b is the amount of enzyme (mL) used to determine the activity. The enzyme activity in the gel was found by dissolving benzidine (30 mg) and NaOAc (1.8 g) in alcohol (30 mL, 50%), adding glacial acetic acid (0.9 mL), and adjusting the volume to 60 mL with H_2O_2 solution (0.004).

Peroxidases were separated by electrophoresis in basic (pH 8.9) PAAG (7.5%) according to Davis [7]. Peroxidase isoenzymes in the gel were detected using benzidine (0.01%) with H_2O_2 (0.005%) in NaOAc buffer at pH 4.7.

Thermal stability of peroxidase was investigated by heating on a water bath at 30, 40, 50, 60, and 70°C in tubes. Every 10 min tubes were selected for determining the peroxidase activity.

Optimum pH values for benzidine oxidation were determined using acetate (pH 3.6, 4.7, 5.5), phosphate (pH 6.0 and 7.0), and Tris-HCl (pH 8.0 and 9.0) buffers.

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