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Characterisation of an acidic peroxidase from papaya (*Carica papaya* L. cv Tainung No. 2) latex and its application in the determination of micromolar hydrogen peroxide in milk

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ABSTRACT

An acidic peroxidase isoform, POD-A, with a molecular mass of 69.4 kDa and an isoelectric point of 3.5 was purified from papaya latex. Using *o*-phenylenediamine (OPD) as a hydrogen donor (citrate–phosphate as pH buffer), the optimum pH for the function of POD-A was 4.6, and the optimum temperature was 50 °C. The peroxidase activity of POD-A toward hydrogen donors was both pH- and concentration-dependent. Under optimal conditions, POD-A catalysed the oxidation of OPD at higher rates than pyrogallol, catechol, quercetin and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The chemical modification reagents *N*-bromosuccinimide and sodium azide significantly inhibited POD-A activity. The results of kinetic studies indicated that POD-A followed a ping-pong mechanism and had a K_m value of 2.8 mM for OPD. Using CPC silica-immobilised POD-A for the determination of micromolar H_2O_2 in milk, the lower limit of determination was 0.1 μM, and the recoveries of added H_2O_2 were 96–109%.

1. Introduction

Peroxidase (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7), an oxidoreductase carrying a b-type haem prosthetic group, uses H_2O_2 to catalyse the oxidation of various electron-donating substrates, such as phenol and aromatic amines. Haem peroxidases can be categorised into two super-families based on their protein sequence similarities. Peroxidases from plants, fungi, and bacteria are structurally related and belong to the plant peroxidase super-family, whereas peroxidases of animal origin constitute a distinct super-family. Plant peroxidases play important roles in the food quality of raw and processed fruits and vegetables, such as the deterioration of flavour, colour, texture and mouthfeel (Ashie, Simpson, & Smith, 1996). Additionally, peroxidases are important in bioanalytical and biotechnological applications, such as enzymatic reagents for clinical diagnosis, degradation of toxic phenols from waste water and drinking water (Akhtar & Husain, 2006; Lai & Lin, 2005), and detergent formulation.

Papaya (*Carica papaya* L.) is widely cultivated in tropical and subtropical regions all around the world for its edible fruits and for the enzymes stored in its laticifers. Laticifers are highly specialised cells, which are anastomosed as a result of the partial hydrolysis of adjacent walls, thereby forming a tube-like network or paracirculatory system through the plant. Latex is the cytoplasm of laticifers. Damaging the papaya tree inevitably severs its laticifers, eliciting an abrupt release of latex. It has been suggested that latex secretion acts as a defence against wounds and/or predators, such as insects and microorganisms. Papaya latex is a thixotropic fluid with a milky appearance that contains approximately 15% dry matter. Forty percent of this dry matter is constituted by enzymes. Papaya latex is a rich source of cysteine endopeptidases and has found many applications in the food and pharmaceutical industries (Iizuka & Aishima, 2000). Papaya latex also contains other enzymes, such as chitinolytic enzymes, glycosidase, glutaminyl cyclotransferase and peroxidase. Several of these enzymes, namely, a lysozyme, a class II chitinase, a β-*N*-acetylhexosaminidase, and a glutaminyl cyclotransferase, have been purified and further characterised (Azarkan et al., 1997; Chen, Chung, Chang, & Chang, 2011; Oberg et al., 1998). However, the peroxidase in the papaya latex has not been purified and characterised to date.

Therefore, in the present study, we purified an acidic peroxidase from papaya (*C. papaya* L. cv. Tainung No. 2) latex, and further characterised its enzymatic properties, including molecular mass,

Abbreviations: *p*APMA, *p*-aminophenylmercuric acetate; BCA, bicinchoninic acid; CBR, Coomassie brilliant blue R-250; CHD, 1,2-cyclohexanedione; DEPC, diethyl pyrocarbonate; DNFB, 2,4-dinitro-1-fluorobenzene; EAM, ethylacetamidate; EDTA, ethylenediaminetetraacetic acid; *p*HMB, *p*-hydroxymercuribenzoate; IEF, isoelectric focusing electrophoresis; PMSF, phenylmethanesulfonyl fluoride; WRK, *N*-ethyl-5-phenylisoazoline-3′-sulfonate (Woodward's reagent K); OPD, *o*-phenylenediamine; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

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isoelectric point, thermal stability, effectors, substrate specificity and kinetic mechanism. Moreover, the purified enzyme was immobilised on a CPC-silica carrier and used for the determination of micromolar H_2O_2 in milk.

2. Materials and methods

2.1. Plant material

Fresh latex of *C. papaya* L. cv Tainung No. 2 was collected from a native specimen grown in Taichung, Taiwan. Upon arrival in the laboratory, the latex was immediately frozen in liquid nitrogen and lyophilised. The lyophilised latex was stored at −20 °C.

2.2. Chemicals

EAM, *p*HMB, DEPC, WRK, PMSF, CHD, DNFB, *p*APMA, OPD, pyrogallol, catechol, ABTS, quercetin and 3-amino-9-ethylcarbazole were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO). Superose 6 HR 10/30, PhastGel IEF 3-9 and isoelectric focusing markers (p*I* 3-10) were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Silane-coated CPC-silica carrier was obtained from Fluka (Buchs, Switzerland). Bio-Lyte 3/10 Ampholyte (40%) was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). All other chemicals were of reagent grade or purer.

2.3. Measurement of peroxidase activity

A 2.65 ml mixture containing 1 ml of 0.1 M sodium citrate–phosphate (McIlvine) buffer (pH 4.6), 0.8 ml of 2.5 mM H_2O_2, 0.8 ml of 10 mM OPD and 0.05 ml of a diluted enzyme solution was incubated at 50 °C for 10 min, and 0.05 ml of 4 N HCl were added to stop the reaction. The quantity of oxidised OPD was calculated from the absorbance measurement at 445 nm using an extinction coefficient (ε) of 11,100 $M^{-1}cm^{-1}$ (Lai, Wang, Chang, & Wang, 2006) according to the Lambert–Beer law. One enzyme unit was defined as the amount of enzyme required to produce one micromole of oxidised OPD per minute.

2.4. Preparation of pAPMA-Sepharose 4B affinity adsorbent

The *p*APMA-Sepharose 4B was prepared by coupling *p*APMA to CNBr-activated Sepharose CL-4B as described previously (Chang et al., 2011).

2.5. Preparation of crude enzyme

One gramme of lyophilised papaya latex was dissolved in 25 ml of 0.14 M sodium phosphate buffer at pH 7.2, and the mixture was stirred for 20 min. Any insoluble substances were removed by centrifugation (6000*g* for 20 min), and the resulting supernatant was designated as crude peroxidase.

2.6. Ammonium sulfate fractionation

Ammonium sulfate was added to the crude peroxidase, and the precipitate that formed upon 0–90% saturation was collected by centrifugation (17,540*g*, 10 min) and dissolved in 10 ml of 25 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.2.

2.7. Removal of cysteine proteases by affinity chromatography

The peroxidase obtained from the 0–90% saturation of $(NH_4)_2SO_4$ was concentrated using ultrafiltration with a 10-kDa molecular weight cutoff (MWCO) membrane (Millipore, Carrigtwohill, Co., Cork, Ireland) and applied to a *p*APMA-Sepharose 4B column (1.0 × 20 cm) that was pre-equilibrated with 25 mM sodium phosphate buffer containing 0.5 M NaCl, pH 7.2, for affinity adsorption of cysteine proteases as described previously (Chang et al., 2011). The fractions exhibiting a non-bound peroxidase activity peak were pooled.

2.8. Rotofor cell preparative isoelectric focusing electrophoresis (IEF)

Preparative IEF was performed using a Rotofor cell (Bio-Rad). The peroxidase obtained from the *p*APMA-Sepharose 4B affinity column was concentrated using ultrafiltration with a 10-kDa MWCO membrane (Millipore). After dialysis against deionised water, the dialysate was brought to a total volume of 58 ml with H_2O and loaded into the Rotofor cell chamber for IEF as described previously (Hsu, Chung, Chang, & Sung, 2012). The fractions containing acidic peroxidase activity (focusing at pH 3–5) were pooled and reloaded into the Rotofor cell chamber for refractionation. After isoelectric focusing electrophoresis, fractions exhibiting an acidic peroxidase activity peak focusing at pH 2.5–4 were pooled.

2.9. Superose 6 HR gel filtration

The refractionated acidic peroxidase isoform obtained from the Rotofor cell IEF was concentrated by ultrafiltration with a 10-kDa MWCO membrane (Millipore), and subsequently purified using a Superose 6 HR column (Pharmacia HiPrep 10/30) that was pre-equilibrated with 25 mM imidazole–HCl buffer, pH 7.4, at a flow rate of 30 ml/h, and 0.5-ml fractions were collected. The purified acidic peroxidase was designated POD-A.

2.10. Determination of the optimum pH and optimum temperature

The optimum pH value for the purified POD was determined using an assay with OPD as the substrate in a universal buffer (Britton and Robinson type; Dawson, Elliott, Elliott, & Jones, 1969) at various pH values between 2 and 10 and at 50 °C. The results are expressed as relative activity percentages that were calculated by dividing the specific activity of the POD at each pH value by the maximum activity. The optimum temperature for activity of the purified enzyme was assayed using the OPD substrate at temperatures between 30 and 80 °C. The results are expressed as the relative activity percentages, which were calculated by computing the ratio of the specific activity at each temperature to the maximum activity identified within the temperature range studied.

2.11. Determination of thermal stability

To assess the thermal stability of POD-A, aliquots of the enzyme solutions were incubated in a thermostatic water bath at different temperatures ranging from 30 to 80 °C. After various time intervals, samples were withdrawn, and the enzyme activity remaining in each sample was measured as described in Section 2.3. The peroxidase activity level from the unheated enzyme solution represented the initial activity (100%). The portion of activity remaining after the heat treatment for different times was calculated. The heat inactivation of the purified enzyme was simulated by first-order kinetics as

$$A = A_0 \times e^{-kt} \tag{1}$$

$$log\frac{A}{A_0} = (\frac{-k}{2.303})t. \tag{2}$$

where A is the remaining peroxidase activity after t of heat treatment. A_0 is the initial peroxidase activity, and k is the reaction rate