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A New Thermostable Peroxidase From Garlic *Allium sativum*

Purification, Biochemical Properties, Immobilization, and Use in H_2O_2 Detection in Milk

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Abstract

Analysis of peroxidase activity by native polyacrylamide gel electrophoresis (PAGE) from a garlic bulb (*Allium sativum* L) extract showed two major activities (designated POX_1 and POX_2). The POX_2 isoenzyme was purified to homogeneity by ammonium sulfate precipitation, gel filtration, and cation-exchange chromatography. The purified enzyme was found to be monomeric with a molecular mass of 36.5 kDa, as determined by sodium dodecyl sulfate-PAGE. The optimum temperature ranged from 25 to 40°C and optimum pH was about 5.0. The apparent K_m values for guaiacol and H_2O_2 were 9.5 and 2 m*M*, respectively. POX_2 appeared highly stable since 50% of its activity was conserved at 50°C for 5 h. Moreover POX_2 was stable over a pH range of 3.5–11.0. Immobilization of POX_2 was achieved by covalent binding of the enzyme to an epoxy-Sepharose matrix. The immobilized enzyme showed great stability toward heat and storage when compared with soluble enzyme. These properties permit the use of this enzyme as a biosensor to detect H_2O_2 in some food components such as milk or its derivatives.

Index Entries: Plant peroxidases; garlic; protein purification; immobilization; kinetic characterization.

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Introduction

Peroxidases (H_2O_2 oxidoreductase; EC 1.11.1.7) are found in many organisms. They are ubiquitous in nature and can be divided into two superfamilies on the basis of amino acid sequence. The first one comprises peroxidases from plant, fungal, and bacteria and the second one is from animals *(1)*. This superfamily has been classified into three classes: Class I is composed of intracellular peroxidases, including cytochrome-*c* peroxidase, ascorbate peroxidase, and the gene-duplicated bacterial catalase-peroxidase. Class II contains secretory fungal enzymes such as manganese and lignin peroxidase, and class III consists of secreted plant peroxidases. In plants, peroxidases are localized in vacuoles, tonoplast, plasmalemma, or cell wall and could have diverse physiologic functions, as evidenced by the presence of several isoenzymes. They are implicated in hormonal regulation *(2)*, stress response *(3)*, pathogen resistance *(4)*, lignification *(5)*, and crosslinkage of cell wall polysaccharides *(6)*.

These enzymes catalyze the oxidation of many organic and inorganic compounds using H_2O_2 or its derivatives. They appear unstable under strong oxidizing conditions *(7)*, and this inhibition is combined with iron oxidation. Various cationic and anionic peroxidase isoforms with a p*I* ranging from 3.5 to 11 and a molecular mass from 28 to 54 kDa have been described in plants. Peroxidases from various sources have interesting characteristics such as heat stability and the ability to yield chromogenic products at low concentration, which makes them suitable for the preparation of enzyme-conjugated antibodies.

Peroxidases have a great diversity of applications, partly owing to their wide substrate specificity and functional diversity. They are useful for industrial and analytic applications where they can be used as catalysts for phenolic resin synthesis *(8)*, as indicators for food processing *(9)*, in the treatment of wastewater by removal of phenols and aromatic amines *(10,11)*, in the biobleaching process *(12)*, in lignin degradation *(13)*, and in the biotransformation of organic compounds *(14)*. Peroxidases are also used as markers in enzyme immunoassays, DNA probes *(15)*, or clinical diagnosis *(16,17)*. They represent the bases of numerous biosensors for the direct determination of many compounds or when coupled with other enzymes in H_2O_2-producing polyenzyme systems *(18)*.

Peroxidase is commonly prepared from horseradish (horseradish peroxidase [HRP]) *Armoracia rusticana* roots. There is a great interest in finding more locally available alternative sources that exhibit properties similar to or better than those of horseradish enzyme.

In this article, we describe the purification and some biochemical properties of a new peroxidase called POX_2 from garlic bulbs. We also discuss enzyme characteristics such as temperature, pH, storage stability, and activity that make POX_2 suitable for use as a biosensor for the detection of H_2O_2 in milk.

Materials and Methods

Garlic and Reagents

Fresh garlic bulbs were obtained from a local market. Only uninjured bulbs were selected. Guaiacol, *o*-dianisidine, diaminobenzidine (DAB), and 3-amino-9-ethyl carbazol were obtained from Sigma. H_2O_2 (30% [v/v]) was supplied by Prolabo. The VA-epoxy-activated matrix, Sartobind Membrane type 18706 and Polymer 39354, were purchased, respectively, from Sartorius® AG and Rieadel-de Haën AG. Peroxidase from horseradish (720 U/mg of protein) was obtained from Fluka Aldrich. All chemicals were of the highest grade commercially available, and all solutions were prepared using deionized water obtained with a milli-Q system.

Preparation of Crude Extract

Garlic bulbs were ground with an electric blender. The smooth paste was crushed in the presence of liquid nitrogen until a fine powder was obtained, which was then homogenized in 25 m*M* Tris-HCl buffer (pH 7.5) containing 0.1 m*M* phenylmethylsulfonyl fluoride and 2 m*M* EDTA at 4°C overnight. After filtration, the extract was centrifuged (4°C, 12,000*g*, 20 min), and the supernatant, considered crude extract, was used for enzyme purification.

Determination of Proteins

Proteins were quantified by the Bradford *(19)* method using bovine serum albumin as the standard or by measurement of the absorbance at 280 nm.

Enzyme Assay

Peroxidase activity was measured by the continuous spectrophotometric quantification of oxidation products of guaiacol at 470 nm in the presence of H_2O_2 and at 25°C (Beckman DU 640 B spectrophotometer, equipped with a temperature regulator). The final reaction mixture contained 0.055% (v/v) or 16.2 m*M* H_2O_2 and 0.3% (v/v) or 26.8 m*M* guaiacol in 10 m*M* sodium acetate buffer, pH 5.0, and suitable amounts of enzyme. All measurements were carried out within 2 min at 10-s intervals. One unit of activity was defined as an increase of 0.1 absorbance/min *(20)*. Peroxidase activity was also assayed by spectrophotometric determination of the increase in absorbance at 470 nm using *o*-dianisidine as reducing substrate.

Purification Procedure

All of the following steps were performed at 4°C, and peroxidase activity was determined using guaiacol as reducing substrate.

Ammonium Sulfate Precipitation

Crude extract proteins were precipitated with ammonium sulfate at 80% saturation. The pellet was collected by centrifugation and dissolved in 25 m*M* Tris-HCl, pH 7.5.

Gel Filtration Chromatography

The concentrated enzyme solution was loaded onto a Sephacryl S-200-HR (Sigma) column (90 × 4 cm) equilibrated with 25 m*M* Tris-HCl buffer, pH 7.5. Elution was performed with the same buffer at a flow rate of 15 mL/h, and 5-mL fractions were collected and monitored for peroxidase activity. Protein content was determined by the measure of absorbance at 280 nm or by the Bradford method.

Cation-Exchange Chromatography

Gel filtration fractions containing peroxidase activity were pooled, their pH was adjusted to 4.5, and they were chromatographed on an SP-Toyopearl-650 C column. The column (7 × 3 cm) was equilibrated with 20 m*M* sodium acetate buffer, pH 4.5. Elution was carried out with 200 mL of NaCl gradient (0–750 m*M*) in the same buffer at a flow rate of 25 mL/h. Two-milliliter fractions were collected and monitored for peroxidase activity and for protein content by measuring the absorbance at 280 nm.

Gel Electrophoresis

Native Cathodic Polyacrylamide Gel Electrophoresis and Peroxidase Staining

Peroxidase was visualized following the method described by Baaziz *(21)* with some modifications by using a Mini-Protean II Cell (Bio-Rad). Slab gels consisted of 12 or 15% acrylamide/bisacrylamide (29:1) in 375 m*M* potassium acetate buffer, pH 6.0. Protein extracts were mixed with 10% glycerol and methylene green in 40 m*M* glycine, 3.5 m*M* acetic acid pH 4.0 buffer. Slab gels 1 mm thick were subjected to electrophoresis at 4°C under nonconstant current.

Peroxidase activity was revealed on the gel as described by Graham and Graham *(22)* with some modifications: The gel was incubated in 0.1 *M* sodium acetate buffer, pH 5. Afterward, it was transposed for 10–15 min in the same buffer containing 0.3% (v/v) fresh guaiacol solution. The gel was reincubated in a buffer solution containing 0.3% (v/v) guaiacol, 0.1% (v/v) H_2O_2, 0.05% (w/v) DAB, or 0.02–0.05% (w/v) 3-amino-9-ethyl carbazol. Activity bands were allowed to develop within 5–10 min, and then the gel was washed with distilled water and scanned.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Molecular mass was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) *(23)* under reducing and nonreducing conditions using Criterion™ Precast Gels (Bio-Rad) with molecular weights markers in the high range from 10 to 200 kDa (Bio-Rad). Samples were run on polyacrylamide (12.5 or 15%) pre-prepared gels (Bio-Rad). Proteins bands were stained by a silver nitrate method *(24)*.

Isoelectric Focusing

Isoelectric focusing (IEF) was performed in a mini IEF Instrument Phast System (Amersham) using pre-prepared phast gels (Amersham) including an ampholine range between 3 and 9 and protein standards (Amersham) in a p*I* range of 3.5–9.5. Protein bands were visualized using a silver technique. The p*I* of the enzyme was determined by comparison with protein standard mixtures: amylglucosidase, p*I* 3.5; methyl red, p*I* 3.75; trypsin inhibitor, p*I* 4.55; β-lactoglobulin A, p*I* 5.2; bovine carbonic anhydrase β, p*I* 5.85; human carbonic anhydrase β, p*I* 6.55; acidic myoglobin, p*I* 6.8; basic myoglobin, p*I* 7.55; acidic lentil lectin, p*I* 8.15; middle lentil lectin, p*I* 8.45; basic lentil lectin, p*I* 8.65; trypsinogen, p*I* 9.5.

Characterization of Enzyme

Optimum pH

POX_2 enzyme activity was tested with the following buffers: 20 m*M* sodium acetate (pH 3.5–5.5), sodium phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0) at ambient temperature (25°C).

Optimum Temperature

Peroxidase activity was determined over a temperature range of 4–75°C at optimum pH.

Optimum Salt Concentration

Activity was examined at various salt concentrations from 0 to 100 m*M* NaCl in 20 m*M* sodium acetate pH 5.0 buffer at ambient temperature (25°C).

pH Stability

Aliquots of purified enzyme were incubated overnight in 20 m*M* buffer at various pH values, from 3.5 to 11.0, and the residual enzymatic activity was determined at optimal conditions.

Thermal Stability

Enzyme solution was heated in 20 m*M* sodium acetate buffer, pH 5.0, at two temperatures (50 and 60°C) for different times. Samples were cooled immediately in ice water. The remaining activity was determined with *o*-dianisidine, which is the more selective substrate used for quantitative studies of heat treatment *(25)*.

Storage Stability

Aliquots of enzyme were kept at 25°C and the activity was assayed every week for 2 mo.

Determination of K_m

The apparent Michaelis constants for guaiacol as reducing substrate and H_2O_2 as oxidant were obtained by varying H_2O_2 concentrations from 0.01 to 0.1% using an enzyme-saturating guaiacol concentration of 0.3%. A guaiacol concentration of 0.02–0.5% was used at an enzyme-saturating

H_2O_2 concentration of 0.05%. This series of experiments was carried at optimal conditions (pH, ionic strength, and temperature) determined previously. The K_m for each substrate was calculated from Lineweaver-Burk plots of $1/V_0$ vs $1/[S_0]$. All assays were performed in duplicate.

Effect of Solvents and Cations

Peroxidase activity was tested with acetone, isopropanol, and ethanol at variable concentrations (2, 5, 15, and 25%) and with Ca^{2+}, Mn^{2+}, Fe^{2+}, and N_i^{2+} at 1 and 5 m*M*.

Detection of H_2O_2 in Milk

Different amounts of H_2O_2 were mixed with a milk sample diluted 10-fold with peroxidase activity buffer in presence of purified enzyme. The minimal H_2O_2 concentration was determined using a guaiacol assay.

Covalent Enzyme Immobilization

POX_2 protein solution collected after cation-exchange chromatography was concentrated by ultrafiltration (Centriplus, Amicon PM10) and washed again in 1 *M* phosphate buffer, pH 7.0. The enzyme was coupled to VA-epoxy-activated carrier (beads and membrane) according to the procedure of Porath et al. *(26)* with some modifications: two hundred milligrams of beads was suspended in 1 mL of enzyme solution (4 mg/mL of protein), or a piece of membrane (4.9 cm^2) was put in 1 mL of enzyme solution (2 mg/mL). These preparations were mixed for 24 h at 4°C and 500 rpm. After immobilization, supports were washed and stored at 4°C in 2 mL of the same buffer until use.

Results and Discussion

Garlic is an interesting medicinal plant that contains several antioxidant substances *(27)*. Zymogram analysis of crude extract peroxidases showed three basic peroxidases (Fig. 1, inset lane 1), and two acidic or neutral peroxidases were shown when performing native anodic PAGE (data not shown).

Purification of Peroxidase

After precipitation of crude garlic extract with ammonium sulfate, the first step was a size-exclusion chromatography through which proteins were separated from yellow pigments. Figure 1 indicates that peroxidase activities were mainly found in a distinct and major peak (peak B) containing POX_2 activity eluted after minor peroxidase activity (peak A).

A native cathodic PAGE profile of the POX_2 step Sephacryl S-200HR is shown in Fig. 1 (inset, lane 2). The pooled fractions of the major peak (peak B) (42–54) were loaded onto the cation-exchange column (TSK gel Sp-Toyopearl-650C). Peroxidase activity was eluted into a single peak at about 200 m*M* NaCl.

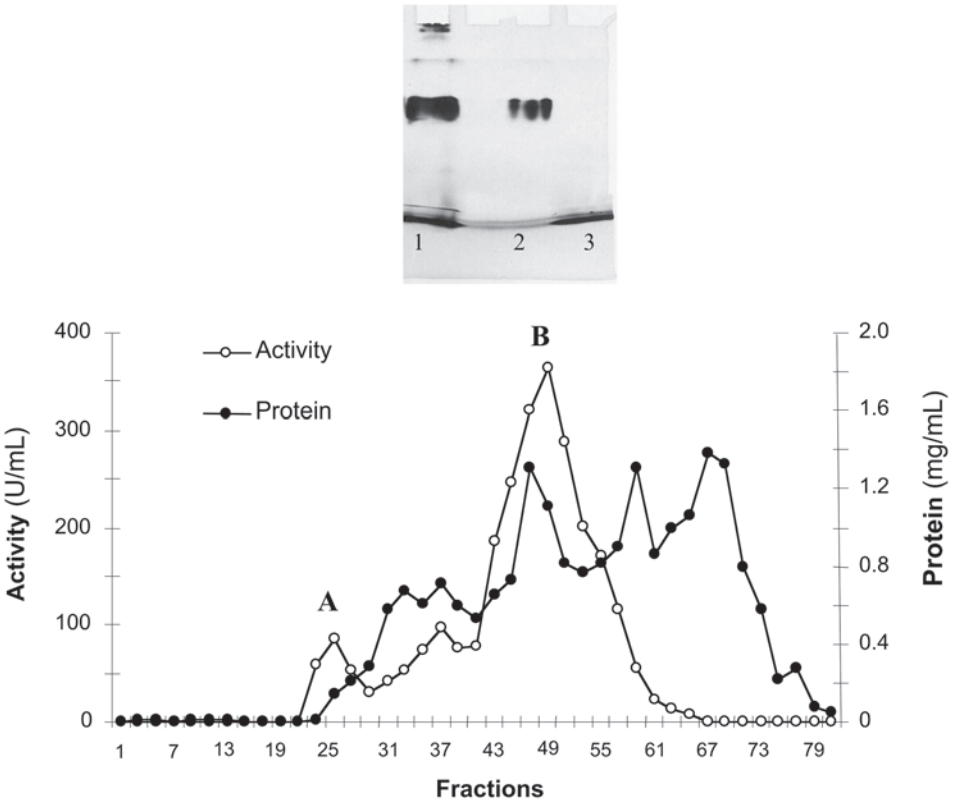

Fig. 1. Elution profile of garlic peroxidase isoenzymes after Sephacryl S-200-HR chromatography. The column was equilibrated with 25 m*M* Tris-Hcl buffer, pH 7.5. Protein content was determined by the Bradford method, and peroxidase activity was measured as described in Materials and Methods. The inset indicates the native cathodic PAGE (12% polyacrylamide gel) profile of garlic peroxidase isoenzymes. Lane 1, crude extract precipitated with ammonium sulfate; lane 2, pool of peak A; lane 3, pool of peak B (POX_2).

One of the two protein peaks was superposed with the peroxidase activity peak (Fig. 2). Active fractions (8–12) were assembled and the pool represents the fraction of POX_2. A native cathodic PAGE profile of the POX_2 step Sp-Toyopearl-650C is shown in Fig. 2 (inset, lanes 3 and 4).

Table 1 summarizes the purification procedure (economical and easy) of POX_2 from garlic bulbs. Cation-exchange chromatography greatly improves purification factor and specific activity. Active fractions from this step were pooled; the preparation corresponded to a pure enzyme that was used in subsequent studies.

Biochemical Characterization of POX_2

Isoperoxidases from garlic have not been reported previously in the literature. We compared our present findings with those obtained from other vegetable sources.

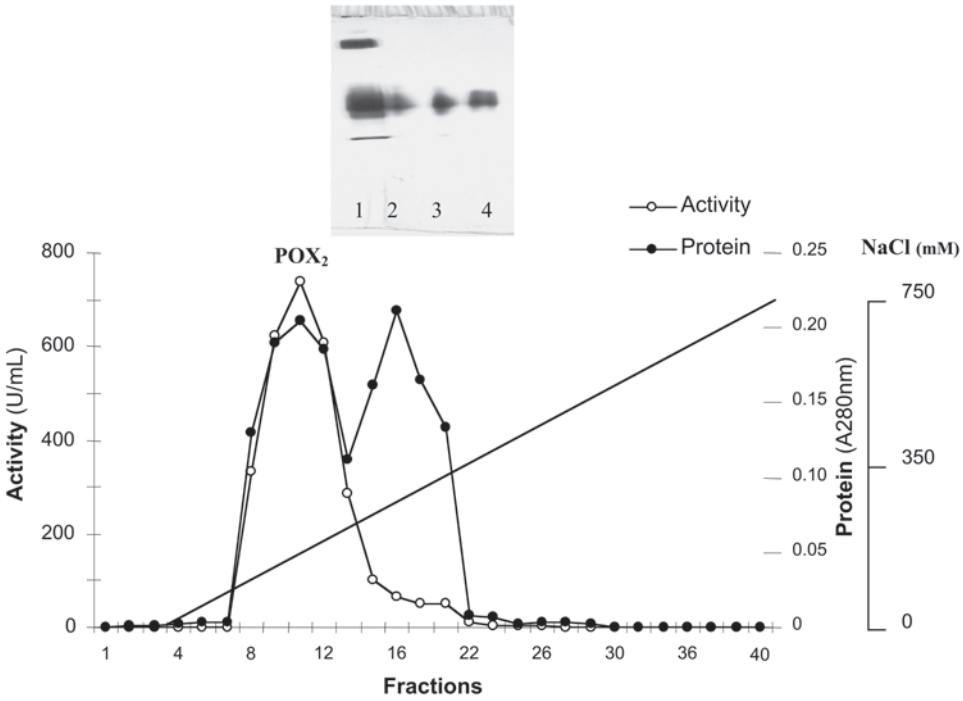

Fig. 2. Elution profile of garlic peroxidase POX_2 after Toyopearl® SP-650C (Tosoh) chromatography. The column was equilibrated with 20 m*M* sodium acetate buffer, pH 4.5. Protein content was determined by measuring the absorbance at 280 nm, and peroxidase activity was measured as described in Materials and Methods. The inset indicates the native cathodic PAGE (15% polyacrylamide gel) profile of garlic isoperoxidases. Lane 1, crude extract precipitated with ammonium sulfate; lane 2, peak B (POX_2) after gel filtration; lanes 3 and 4, POX_2 fraction after cation exchange.

Table 1
Summary of Purification Procedure for Garlic Peroxidase (POX_2)

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude extract	497	50,042	101	1.00	100
Ammonium sulfate precipitation	177.5	39,375	222	2.20	79
Sephacryl S-200 HR	63.2	21,715	344	3.41	44
Sp-Toyopearl-650C	0.27	3645	13,860	138	7.3

Structural Properties

The molecular mass determined by SDS-PAGE in the presence of 2-β-mercaptoethanol was estimated at 36.5 kDa (Fig. 3A) and was similar to that obtained under no reducing conditions, with no additional bands. This indicates that the protein consisted of a single polypeptide chain.

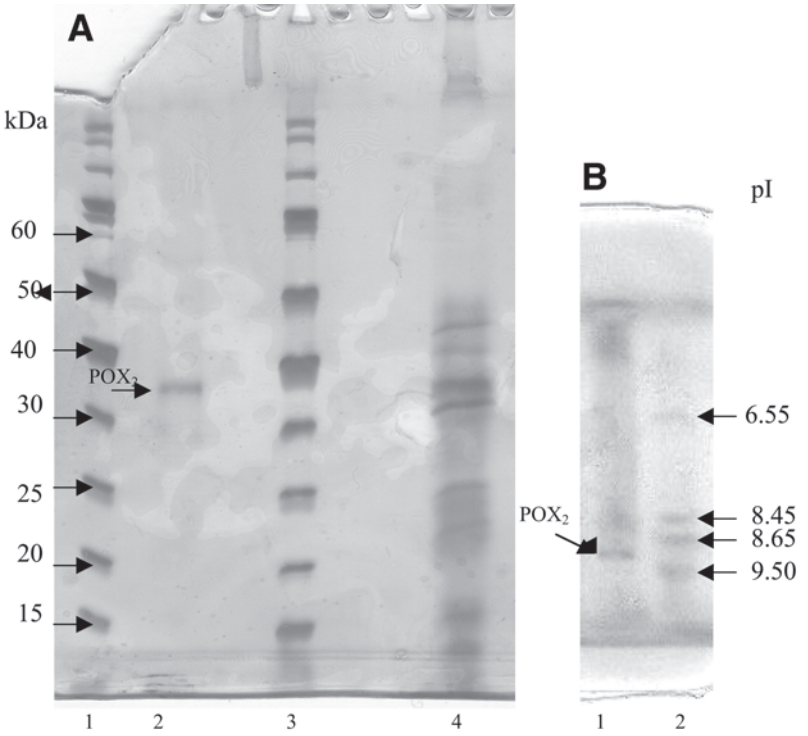

Fig. 3. **(A)** SDS-PAGE analysis of garlic peroxidase isoenzymes (12.5% polyacrylamide gel) stained with silver nitrate: lanes 1 and 3, molecular weight markers; lane 2, POX_2 fraction from cation-exchange chromatography; lane 4, peak B (POX_2) from gel filtration. **(B)** IEF gel of POX_2 fraction revealed with silver technique. Lane 1, POX_2 issued from cation-exchange chromatography; lane 2, p*I* standards.

The molecular mass value agrees with a range of 30–54 kDa from a variety of fruits and vegetables *(28)* as reported for *Cucumis sativum (29)*. However, this value was lower than those described for oil palm (*Elaies guinensis*) (57 kDa) *(30)* and turnip (*Brassica napus*) (39.2–42.5 kDa) *(31)* but higher than for alga (*Galdiera partita*) peroxidase (28 kDa) *(32)*.

After analysis by IEF, POX_2 appeared as a single band with a p*I* of about 9 according to the measured pH gradient along the gel (Fig. 3B). Basic peroxidases have been found in turnip roots *(17,31)*, *Aloe barbadensis (33)*, *C. sativus (29)*, and *Camellia sinensis (34)*. The p*I* of POX_2 is the same as that reported for green peas (*Pisum sativum*) *(35)*.

Regarding physiologic roles, alkaline peroxidases are implicated in plant hormone regulation, and acidic peroxidases participate in the last stage of lignin biosynthesis *(36)*.

Native cathodic PAGE, IEF, and SDS-PAGE strongly suggest that the POX_2 fraction was purified to homogeneity. The Toyopearl® SP-650C (Tosoh) POX_2 fraction was then used to investigate the catalytic properties.

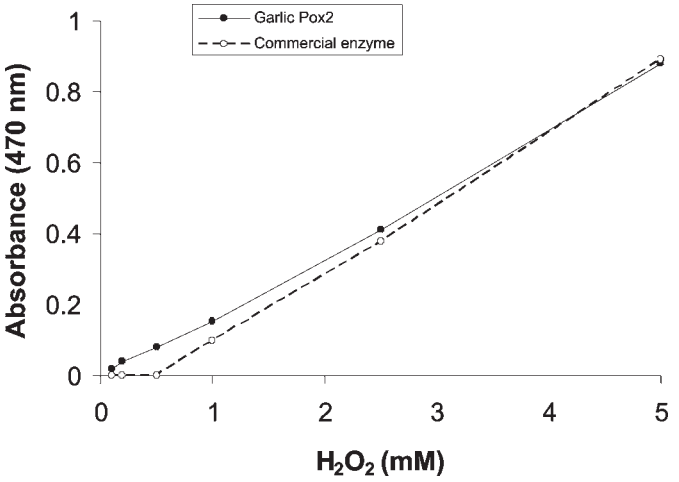

Fig. 4. Differential detection of H_2O_2 in milk by POX_2 (●) or commercial peroxidase (○). Peroxidase activity was monitored by measuring the absorbance at 470 nm with increasing concentrations of H_2O_2 from 0.25 to 5 m*M* as described in Materials and Methods.

Kinetics and Effects of Inhibitors and Bivalent Ions

The optimum pH for the reaction with guaiacol was about 5.0. This value is similar to that reported for cationic and anionic isoperoxidases from turnip *(31)* but differs slightly from the value for *A. barbadensis* (pH 6.0) *(33)*. However, optimum pH is not a suitable factor for comparison because peroxidase activity depends on the reducer substrate and buffer.

The optimum temperature range of 35–40°C is not in agreement with temperatures reported for isoenzymes C2 (45°C) and C3 (55°C) but agrees with that for fraction C1 (40°C) of turnip *(31)*.

The guaiacol K_m value (9.5 m*M*) was slightly higher than the value (6.9 m*M*) reported for palm *Roystonea regia* major isoperoxidase II *(37)*. The POX_2 apparent K_m toward H_2O_2 (2 m*M*) was much lower than that found for *R. regia* isoperoxidase (15 m*M*) *(37)*.

The study of temperature stability showed that POX_2 retained full activity after 2 h of incubation at 50°C (Fig. 4), which indicates a greater temperature stability when compared with peroxidases C_1 (50% at 3 min), C_2 (50% at 4 min), C_3 (50% at 6 min), and A_2 (50% at 10 min) from *B. napus (31,38)*. POX_2 was also more temperature stable than other peroxidases, such as BP1 from barley grain *(39)*.

At 60°C, POX_2 retained 50% of its activity after 15 min (Fig. 4) and was more thermostable than A_2 peroxidase from turnip, which lost 90% for 10 min *(38)*, and HRP C, the commercial isoenzyme, which lost 50% at 57°C for 10 min *(25)*. By contrast, POX_2 was less temperature stable than soybean peroxidase, which showed a half-life over 12 h at 80°C *(40)*, and palm peroxidase isoenzyme, which was active until 85°C *(37)*.

Table 2
Effects of Bivalent Ions and Solvents
on Peroxidase Activity (POX_2) From Garlic[a]

	Relative activity			
Solvent	2%	5%	15%	25%
Ethanol	93.3	79.4	43.4	27.7
Acetone	93.3	84.9	72.5	57.8
Isopropanol	78.8	76.1	65.3	21.7
Cation	1 m*M*	5 m*M*		
Fe^{2+}	34	25.4		
Ca^{2+}	30	21.3		
Zn^{2+}	64	47		
Ni^{2+}	44.2	43		
Mn^{2+}	57.7	44.5		

[a]Peroxidase activity was determined as described in Materials and Methods. After preincubation at 4°C for 15 min with individual solvents or cations, the remaining activity was expressed as the percentage of initial enzyme activity in the absence of solvents or cations.

POX_2 is stable at a pH range of 5.0–11.0, with 75% of retained activity at pH 3.5. The enzyme appears to be stable against alkaline denaturation, but soybean peroxidase is more stable against both acidic *(41)* and alkaline denaturation *(42)*.

The effects of various inhibitors and metal ions were determined using the guaiacol-H_2O_2 system: POX_2 was inhibited by all tested metallic ions (Fe^{2+} Zn^{2+} Ni^{2+}, Mn^{2+}, and Ca^{2+}) at 5 m*M* (Table 2). Inhibition with isopropanol and ethanol (25%) was two times higher (residual activity of 24%) than in the presence of acetone (58% of relative activity) at the same concentration (Table 2).

Detection of H_2O_2 in Milk

To check the enzymatic detection of H_2O_2 at low concentrations, the sensitivity of POX_2 to H_2O_2 was tested in milk. In some cases, H_2O_2 was added fraudulently to milk. The results showed that POX_2 was able to detect the peroxide at 1 to 2 m*M* (Fig. 4). A similar result was obtained with a commercial preparation of HRP. Consequently, POX_2 isolated from a garlic soluble fraction could be used to detect H_2O_2 in food components.

Immobilization, Temperature, and Storage Stability

Interestingly, comparison of temperature stability between soluble and immobilized enzyme showed that the immobilized form of POX_2 retained full activity for 35 min at 60°C, whereas the free enzyme lost about 78% in the same conditions (Fig. 5). In addition, soluble POX_2 isoenzyme retained full activity for at least 6 wk during storage at 25°C and lost

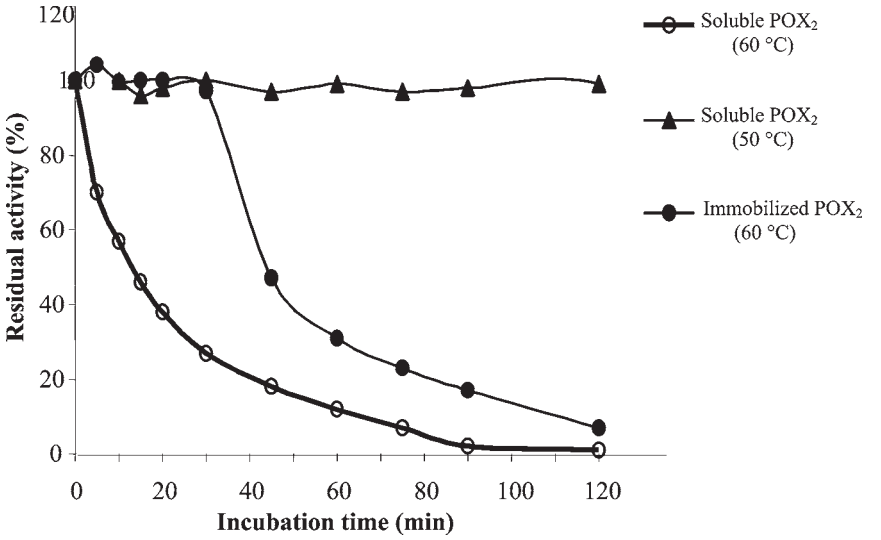

Fig. 5. Effect of temperature on POX_2 activity: heat inactivation of soluble POX_2 at 50°C (▲) or 60°C (○) and immobilized POX_2 at 60°C (●). Remaining activity was expressed as the percentage of initial enzyme activity.

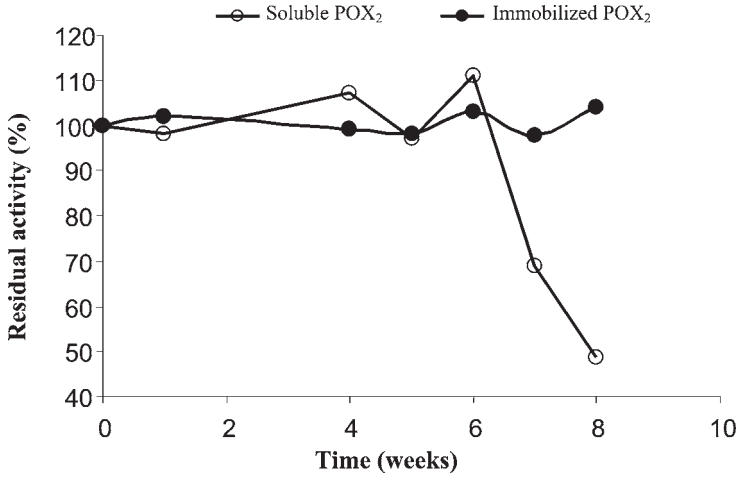

Fig. 6. Effect of duration of storage on POX_2 activity: soluble enzyme (○) or immobilized POX_2 (●). The remaining activity was expressed as the percentage of initial enzyme activity.

50% of its activity after 2 mo, whereas the immobilized enzyme retained complete activity after 2 mo of storage at 25°C (Fig. 6).

Conclusion

The interesting characteristics of the kinetic properties of POX_2 enzyme, temperature, pH, storage stability, and activity, allow it to be

applied successfully as a biosensor to detect H_2O_2 at low concentrations. Moreover, POX_2 should be used in wastewater detoxification or as a biosensor for the rapid detection of peroxides in food and beverages. Furthermore, immobilization of peroxidase activity could provide access to a continuous system for testing peroxides in agroindustrial components.

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