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Purification and partial characterization of a thermostable peroxidase isoenzyme from Bambara groundnut (*Vigna subterranea* L. Verdc) seedlings

Yves Mann Elate Lea Mbassi *, Marie-Solange Evehe Bebandoue and Wilfred Fon Mbacham

Laboratory for Public Health Biotechnology, P.O.Box 8094, Department of Biochemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon.

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Abstract

Thermostable isoperoxidases, especially peroxidase A6, have already been found in seedlings of *Vigna sp*, indicating their potential for biotechnological applications. In this study, peroxidase A6 was purified from the roots of Bambara groundnut (*Vigna subterranea* L. Verdc) seedlings by a combination of gel filtration on Sephadex G-100, heat treatment, and ion exchange chromatography on CM cellulose and DEAE cellulose. The 41-kDa enzyme shows high catalytic efficiency in the oxidation of O-dianisidine, ABTS, TMB, DAB and OPD at acidic pH optimum and the reduction of H_2O_2. It has an optimum temperature of activity of 60 °C and a calculated activation energy of 221.5 KJ/mol for its thermal inactivation at pH 8. Mg^{2+} inhibits the enzyme. Ca^{2+} strongly increases its thermal stability, while Mn^{2+} and Zn^{2+} decrease it. The enzyme is inhibited by sodium azide at concentrations above 1 µM, with an IC_{50} value of about 10 µM. This inhibition, in addition to the RZ value of 2.4 (A_{403nm}/A_{280nm}), confirms the presence of a haem group in the active site, which is common for class III plant peroxidases. Due to its unique catalytic and thermal properties, peroxidase A6 has the potential to be a valuable tool for various biotechnological applications, especially for enzyme immunoassays and clinical diagnosis.

Keywords: Bambara groundnut; Isoperoxidases; Catalytic efficiency; Thermal stability

1. Introduction

Peroxidases are widely used in biotechnology because of their wide distribution throughout the living world and the wide variety of substrates they can bind to. Peroxidases find extensive use in processes that occur at somewhat high temperatures. This is the case for wastewater treatment, which takes place at high temperatures for hours, or hydrogen peroxide biosensors, where peroxidases must work for hours at about 37 °C [1, 2]. The decreased danger of contamination by typical mesophiles is a very real benefit of conducting biotransformations at high temperatures. Because of the substrates' increased diffusion coefficient and decreased viscosity, high temperatures also speed up reactions, which is advantageous for production yields [3]. However, the majority of enzymes become unstable at high temperatures. While most peroxidases that have been investigated thus far exhibit optimal activity in the range of 30 to 50 °C [4, 5, 6], very few of them can withstand exposure to those temperatures for extended periods. Additionally, horseradish peroxidase, whose heat stability is weak, is currently the main peroxidase that is commercially accessible. Finding peroxidases with improved stability and diverse substrate specificity is therefore necessary. This would enhance immunoenzymatic analytical kits that use peroxidases as immunoconjugates and encourage the development of novel analytical techniques and industrial processes.

However, the fact that thermostable enzymes are generally less flexible at room temperature and reach their maximum catalytic potential only at temperatures over 50 °C or 70 °C presents an inherent challenge with them [7]. This is a major disadvantage for applications such as enzyme immunoassays, which are done at ambient temperature. Useful

* Corresponding author: Yves Mann Elate Lea Mbassi

thermostable enzymes for these diagnostic applications are those that have also great activity at moderate temperatures, conditions compatible with the biological activity and the stability of the other reagents implied in the analysis.

Prior research has identified thermostable peroxidase isoenzymes in *Vigna sp*. (Bambara groundnut) Seedlings. After being exposed to 80 °C and 85 °C for 10 minutes, these peroxidases still exhibited significant activity. Additionally, one of these isoperoxidases had the benefit of being more active in these seedlings than the others. For a comprehensive investigation of its biotechnological potential, including its kinetics towards different peroxidase substrates frequently utilized in immunoassays, and its behavior concerning different heat treatments, its isolation from this plant is thus required. The purpose of this work is to purify the peroxidase isoenzyme known as peroxidase A6 in previous research [8], and to investigate its substrate specificity and its stability in relation to heat and salts.

2. Material and methods

2.1. Chemicals

The following reagents were from SIGMA or SIGMA Aldrich: ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), O-dianisidine, OPD (orthophenylene diamine), TMB (3,3',5,5' tetramethyl benzidine), DAB (3,3'-diamino benzidine), 30 % hydrogen peroxide, PMSF (phenylmethylsulfonylfluoride), EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), TEMED (N,N,N',N'- tetramethyl éthylène diamine), monobasic and dibasic sodium phosphate, sodium acetate, tris base, HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)), sodium hydroxide, boric acid, Sephadex G-100, carboxy-methyl cellulose, diethylamino-ethyl cellulose, polyvinylpyrrolidone, glycerol, coomassie brilliant blue G and R-250, ammonium persulfate, SDS (sodium dodecyl sulfate), sodium azide. Some others were from MERCK (imidazole, sodium chloride), FLUKA (85 % orthophosphoric acid, acrylamide, bis-acrylamide), BAKER (bromophenol blue), ROTH (β-mercaptoethanol), QUIMICA DE MONTCADA SA (acetic acid), BIORAD ("Silver stain plus" kit), CARLO ERBA (37% Hydrogen chloride), New England Biolabs (BSA, prestained protein markers broad range 7708S and unstained protein markers 7702S), USB (Glycin), Amersham Biosciences (Sephadex G-25).

Solvents (methanol, ethanol, and acetone) were purchased from local suppliers. Distilled water was used to prepare aqueous solutions.

2.2. Plant material

Seeds of a Bambara groundnut landrace with white seeds and a slightly black color around the hilum, cultivated in the northern part of Cameroon were purchased at a local market of Yaoundé.

2.3. Germination procedure

The process that was previously outlined [9] was followed for seeds to germinate. After sterilizing the seeds for 20 minutes with 10 % sodium hypochlorite (v/v), the seeds were rinsed four times with distilled water, with the water volume in each washing being double that of the seeds in the container. In Petri dishes, seeds were arranged 15 seeds per dish on top of a layer of moistened filter paper. Every three days, the latter was kept moist with distilled water and stored at room temperature in the dark. The seedlings were gathered after nine days and kept at -20 °C until further used.

2.4. Enzyme assays

Peroxidase activity was determined in a 2 ml reaction mixture constituted by 100 µl of 0.5 mM hydrogen peroxide and 100 µl of either 1 mM O-dianisidine (3,3'-dimethoxy-benzidine) or 1 mM ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) in 100 mM acetate buffer pH 5. The reaction was initiated by the addition of hydrogen peroxide, and oxidation of the reducing substrate was followed by the increase in absorbance at 460 nm for O-dianisidine or 414 nm for ABTS. One unit of peroxidase activity was defined as the amount of enzyme that oxidizes 1 µmol of O-dianisidine per minute at room temperature under the above conditions.

2.5. Protein determination

The protein concentration at the various stages of purification was determined by the Bradford method [10] with bovine serum albumin as a standard.

2.6. Purification procedure

Roots of 9-day-old Bambara groundnut seedlings were ground with a mortar and pestle in 50 mM ice-cold phosphate buffer pH 7 containing 5 mM EDTA, 1 mM EGTA, and 1 mM PMSF. Extraction procedures were done on ice or at 4-8 °C. The ratio of buffer volume per gram of plant material was 5:1. The homogenate was filtered through filter paper (Whatman #3) and the filtrate was centrifuged at 15000 g for 20 minutes. The supernatant was mixed with 1.5 volumes of ice-cold acetone and kept at -20 °C overnight. This mixture was thereafter distributed in microfuge tubes and centrifuged at 15000 g for 5 min. The supernatant was discarded and the pellet was resuspended in a minimal volume of 100 mM phosphate buffer pH 7. The resuspended pellet was again centrifuged at 15000 g for 5 min to eliminate denatured proteins; this supernatant was then incubated at 55 °C for 10 min in a water bath previously thermostated at this temperature. Then it was cooled in ice, and centrifuged at 15000 g for 20 min. The supernatant (7 ml) was subjected to a gel filtration chromatography on Sephadex G-100 (40 cm × 2 cm) previously equilibrated with 3 liters of 100 mM phosphate buffer pH 7. Elution was done with the same buffer, 1.5 ml fractions were collected and their peroxidase activity was tested by using O-dianisidine and H_2O_2. The active fractions constituted a broad peak in the chromatogram, and so were pooled and equilibrated with 10 mM acetate buffer pH 5 by gel filtration on a column of Sephadex G-25 (4 cm × 1.5 cm) previously washed with the same buffer. The extract collected after chromatography on Sephadex G-25 (67 ml) was applied on a column of CM-cellulose (40 cm × 2 cm) equilibrated with 5 column volumes of 10 mM acetate buffer pH 5. Stepwise elution was carried out by increasing concentrations of the same buffer (10 mM, 100 mM, and 1 M). Fractions of 1.5 ml were collected, and their activity was tested with O-dianisidine and H_2O_2 as substrates. For each buffer concentration, the elution was carried out until fractions with zero activity were obtained after the collection of active ones. The active fractions were pooled by peaks of activity, then, to identify the corresponding isoenzymes, the various groups were subjected to native electrophoresis on polyacrylamide gel in the 2 directions of migration. A slow-migrating anodic isoperoxidase (peroxidase A6) was equilibrated in 10 mM Tris buffer pH 8 by loading on Sephadex G-25 previously washed with the same buffer. The active fractions collected after chromatography on Sephadex G-25 were pooled and loaded on a DEAE-cellulose column equilibrated with 10 column volumes of 10 mM Tris buffer pH 8. After loading the sample, the column was washed with the equilibration buffer, and then peroxidases were eluted with 100 mM Tris buffer pH 8. Fractions of 1.5 ml were collected, and their activity was measured with O-dianisidine and H_2O_2 as substrates. Fractions corresponding to the main peak of activity were pooled and concentrated by dialysis against 15 % polyvinyl pyrrolidone. The fractions corresponding to the first broad peak obtained after chromatography on CM-cellulose were also pooled and submitted to chromatography on DEAE-cellulose under the same conditions described for peroxidase A6 to separate the mixture of isoenzymes that it was constituted.

2.7. Electrophoresis and gel staining

Non-denaturing polyacrylamide gel electrophoresis was performed in 6 % polyacrylamide gel with the continuous buffer system Imidazole/HEPES pH 7.4 described by McLellan [11]. Electrophoresis was carried out toward the anode to separate anionic isoperoxidases and toward the cathode to separate cationic isoperoxidases. The gel was run at a constant voltage of 200 V until the front dye reached the bottom. Gels were stained with a mixture of 0.025 % O-dianisidine and 0.2 mM H_2O_2 in 100 mM acetate buffer pH 5. A purified isoperoxidase was subjected to a denaturing polyacrylamide gel electrophoresis in the presence of SDS. The discontinuous system of Laemmli [12] was used for this purpose. One hundred and fifty microliters of purified extract were denatured by heating at 100 °C for 5 min in 30 µl of 6 X sample loading buffer (3 mg of SDS, 9 µl glycerol, 21 µl of 4 X stacking gel buffer, 0.03 % bromophenol blue). The migration was then carried out on a gel made up of a 4 % stacking gel whose pH is 6.8 and a 12 % resolving gel of pH 8.8. The electrode buffer consisted of Tris/glycine buffer pH 8.3. The migration was done initially at 150 V until the bromophenol blue reached the resolving gel, and then the voltage was increased to 200 V, the migration running until the bromophenol blue reached the lower end of the resolving gel. The revelation of the bands was done by the Coomassie blue staining and the silver nitrate methods.

2.8. Substrate specificity

2.8.1. Determination of optimum pH for catalysis of diverse substrates

Peroxidases being able to act on various reducing substrates, we chose 5 of the most used substrates in the biomedical applications of these enzymes: O-dianisidine (3,3'-dimethoxy-benzidine), ABTS (2,2' azino-(a)-(3-ethylbenzothiazoline-6-sulfonic acid)), TMB (3,3',5,5' tetramethyl benzidine), DAB (3,3' -diamino benzidine) and OPD (orthophenylene diamine). We studied the pH effect on the catalytic activity of the purified isoperoxidase. To this end, nine buffer solutions at the concentration of 25 mM were prepared: Glycine/HCl pH 2 and pH 3, Acetate/HCl pH 4 and pH 5, $H_2PO_4^-$ /HPO_4^{2-} pH 6, pH 7 and pH 8, boric acid /NaOH pH 9 and pH 10. For each pH, five microliters of purified enzyme (0.044 µg) were incubated in a closed tube with 1.8 ml of buffer and 100 µl of 1 mM reducing substrate for about thirty minutes at room temperature. The solution was transferred in a spectrophotometer cell. One hundred microliters of 1 mM H_2O_2 were then added, the mixture was briefly homogenized with the Vortex mixer, and the variation of absorbance was read

at 460 nm for O-dianisidine ($\varepsilon_{460\ nm}$= 11.3 mM^{-1} cm^{-1}), 414 nm for ABTS ($\varepsilon_{414\ nm}$= 31.1 $mM^{-1}cm^{-1}$), 655 nm for TMB ($\varepsilon_{655\ nm}$= 5400 M^{-1} cm^{-1}), 405 nm for DAB ($\varepsilon_{405\ nm}$= 5500 M^{-1} cm^{-1}) and 450 nm for OPD ($\varepsilon_{450\ nm}$= 1,05 mM^{-1} cm^{-1}).

2.8.2. Determination of kinetic parameters for catalysis of diverse substrates

Kinetic parameters were determined at the optimal pH obtained in our study, for the five reducing substrates (ABTS, O-dianisidine, OPD, TMB, DAB) and H_2O_2 (by using ABTS at its optimal pH as reducing substrate). For each reducing substrate, the concentration of H_2O_2 was maintained at 30 mM while that of the reducing substrate varied from 1 to 20 mM. For the determination of the kinetic parameters of H_2O_2, the concentration of the reducing substrate was maintained to 9 mM and that of H_2O_2 varied from 0.4 to 4 mM. The double-reciprocal plot method of Lineweaver and Burk [13] was used to obtain the apparent Km and Vmax.

2.9. Optimal temperature

The activity of the purified isoperoxidase was tested at various reaction temperatures (30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C). The method consisted of incubating in a water bath for at least 30 min a mixture made up of 1800 µl of 100 mM acetate buffer pH 5, 100 µl of 1 mM O-dianisidine, and 100 µl of 0.5 mM H_2O_2 at each of these temperatures. The solution thus made up was then quickly transferred in a spectrophotometer cell containing 5 µl of the pure enzyme, and the variation of absorbance was immediately determined at 460 nm every 3 s for the first 15 s. Such a short period was chosen to minimize the denaturation of enzyme at high temperatures during activity measurements.

2.10. Thermal stability assays

2.10.1. Effect of high temperatures

Two hundred microliters of pure enzyme in 100 mM Tris/HCl buffer pH 8 were introduced into a microfuge tube (Eppendorf type) narrowly closed and were incubated for 1 hour in a water bath previously thermostated at high temperatures (70 °C, 80 °C and 90 °C). For each temperature, 10 µl aliquots were collected every 10 min and cooled immediately in ice. The enzymatic reaction was carried out by using 1 mM O-dianisidine and 0.5 mM H_2O_2. The residual activity was calculated as the ratio of activity of the test sample to that of the control which is an extract not heated. In parallel, 100 µl of the enzyme were incubated for 4 weeks at 50 °C, measurements of peroxidase activity being made every week following the same procedure as that described for higher temperatures.

The half-life ($t_{1/2}$) and apparent first-order rate constants of enzyme inactivation (k) at each temperature were obtained from the log (A/Ao) vs. time plots where Ao is the initial enzyme activity, A is the residual activity after heating for time t. The activation energy (E) for thermal denaturation was calculated from the slope (-E/R) of the Arrhenius plot, whose equation is as follows:

$$\ln k = \ln A - \frac{E}{RT}$$

In this formula, E is defined by Arrhenius as the energy differential between the reactants and an active species, and k is a first-order kinetic constant that varies with temperature. Thus, the term E is known as the activation energy. The term A is the pre-exponential factor. This is related to the frequency of molecular collisions in the collision theory and to the entropy term in the transition state theory. The activation energy is the energy barrier that the reactants must overcome to react. Therefore, the high activation energy of a thermal denaturation reaction is a characteristic of most thermostable enzymes.

2.10.2. Storage stability at room temperature

One milliliter of purified enzyme was incubated in a narrowly closed tube for 6 months at room temperature (23-26 °C on average). Five microliter aliquots were withdrawn each month and directly used for tests of activity. The reaction medium initially consisted of 5 µl of enzyme extract, 100 µl of 1 mM O-dianisidine, and 1800 µl of 100 mM acetate buffer pH 5, the reaction being started by 100 µl of 0.5 mM hydrogen peroxide. The residual activity was calculated as the ratio of activity of the test sample to that of the control which is an extract stored at - 20°C.

2.11. Effect of metal ions on the activity and thermal stability of peroxidase A6

We tested the effect of some metal salts ($MgCl_2$, $MnCl_2$, $ZnCl_2$, and $CaCl_2$) on the activity and thermostability of the isoperoxydase that we purified in this study. Five microliters of enzyme were incubated in the presence and absence of

100 µl of a 1 M aqueous solution of each of these salts, volume was completed to 1 ml with 100 mM acetate buffer pH 5. The incubation was done at ambient temperature (~25 °C) and 80 °C during 10 minutes. The content of the tube was then transferred in a spectrophotometer cuvette containing 1 ml of 100 mM acetate buffer pH 5 and 100 µl of 0.1 % TMB. The reaction was started with 50 µl of 0.1 % H_2O_2. Relative activities at room temperature were calculated by comparing the activities obtained in the presence of salts to those of control. To evaluate the effect of metal salts on thermal stability, the residual activities obtained after incubation at 80 °C for 10 min were divided by the relative activities calculated after incubation at room temperature.

2.12. Study of the inhibitory effect of sodium azide

Small molecules such as carbon monoxide, cyanides, fluorides, and azide can bind to the peroxidase heme iron atom through six coordination bonds. Sodium azide is an irreversible inhibitor of heme peroxidases. The initial goal of our study being to seek a peroxidase joining the ideal characteristics for an application in clinical diagnosis and immunoassays, we evaluated the effect of sodium azide on activity of the purified peroxidase, since commercial preparations of antibodies used for the coupling of enzymes often contain sodium azide as preservative of microbial contamination. In addition, the inhibition of peroxidase activity by sodium azide would confirm the nature of the elements present at its active site. For that, we prepared solutions of this compound at variable concentrations (from 0 to 10 M). One hundred microliters of an aqueous solution of this compound were incubated with 5 µl of enzyme for about 30 minutes in a spectrophotometer cuvette, then to this mixture, were added 100 µl of 1 mM O-dianisidine and 100 µl of 0.5 mM H_2O_2. The variation of absorbance was read as previously described.

2.13. Statistical analysis

Results are expressed as means ± standard deviations for the indicated number of experiments. Some data were analyzed by the Mann-Whitney-Wilcoxon test. *P*-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Isolation of peroxidase A6

The purification scheme of peroxidase A6 is presented in Table 1. After the precipitation of proteins by acetone, they were submitted to a 10-minute heat treatment at 55 °C to eliminate any contaminating proteins of low thermal stability, and the results show a very slight increase in the specific activity.

Table 1 Purification scheme of peroxidase A6

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	758	586	0.773	1	100
Acetone precipitation	24.6	168	6.83	8.83	28.67
Heating at 55°C	20.8	143	6.85	8.86	24.37
Gel filtration	3.076	161	52.28	67.61	27.44
CM-cellulose	0.385	20.24	52.64	68.08	3.45
DEAE-cellulose	0.082	10.04	122.29	158	1.72

The peroxidase extract was subjected to gel filtration on Sephadex G-100, after which only one broad peak of activity was detected (Fig. 1), suggesting that peroxidases of Bambara groundnut seedlings have nearby molecular weights.

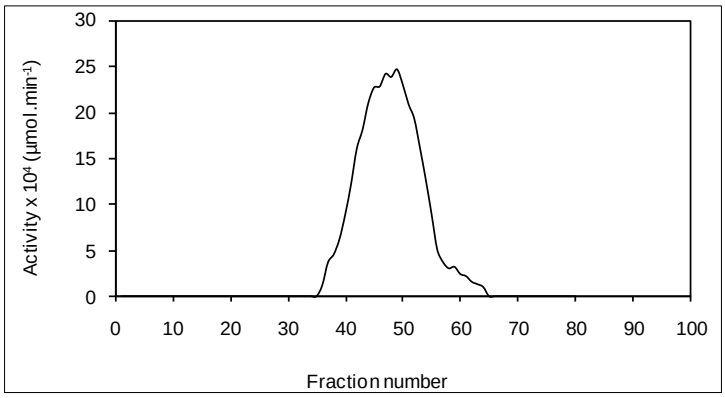

Figure 1 Elution profile after gel filtration on Sephadex G-100. The elution buffer was 0.1 M phosphate buffer pH 7

Following their separation by ion exchange chromatography on CM-cellulose, a group of close peaks between 2 broad peaks of activity was obtained (Fig. 2). The composition of these peaks was analyzed by native-PAGE (data not shown), and allowed the following observations: the first broad peak, eluted by washing of the column corresponded to a mixture of anionic isoperoxidases not retained by the matrix; the group of close peaks corresponded to a mixture containing mainly isoenzyme A6, which was eluted by 100 mM acetate buffer; the last peak represented the cationic peroxidases, eluted by 1 M acetate buffer.

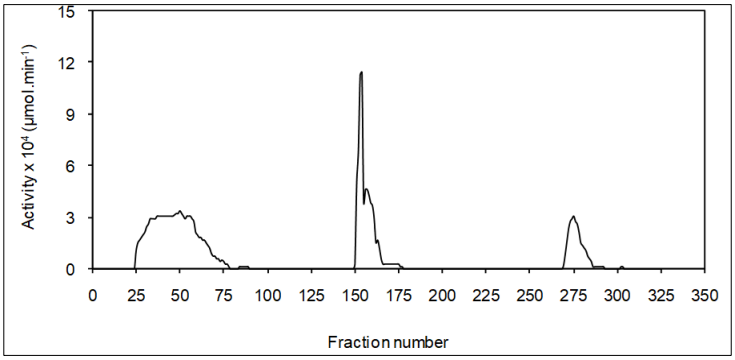

Figure 2 Elution profile of peroxidase extract after ion exchange chromatography on CM-cellulose. Stepwise elution was carried out by increasing concentrations of acetate buffer pH 5 (10 mM, 100 mM, and 0.5 M)

The fractions of the major peak of the group of close peaks were pooled and subjected to an ion exchange chromatography on DEAE-cellulose, to eliminate any trace from minor peroxidases. The result shows a high peak of activity and some minor peaks after the major peak (Fig. 3).

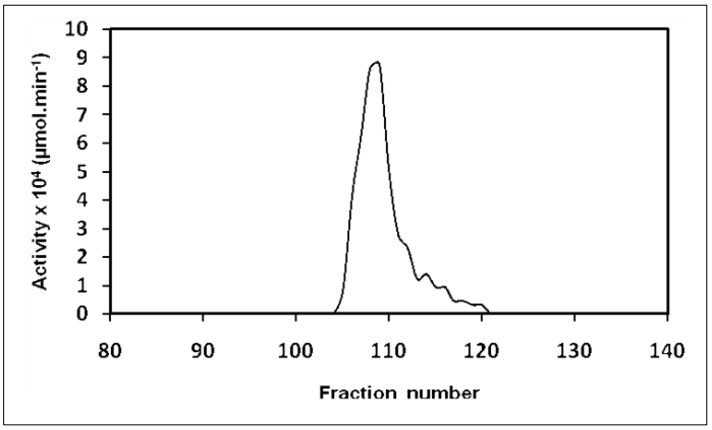

Figure 3 Elution profile of peroxidase A6 after ion exchange chromatography on DEAE-cellulose. Elution was carried out with 100 mM Tris buffer pH 8 after a washing step with 10 mM Tris buffer pH 8

Fractions 105 to 111, corresponding probably to a single isoenzyme, peroxidase A6, were pooled. The fractions not retained by CM-cellulose were also submitted to an anion exchange chromatography on DEAE-cellulose, and two peaks of activity were obtained (data not shown) and fractions corresponding to each peak were pooled.

The purification of peroxidase A6 however was achieved with poor yield. This poor yield is explained by the fact that seedlings of Bambara groundnut contain a multitude of isoperoxidases and that the activity of the crude extract is due to the sum of the individual activities of these isoenzymes, thus, though relatively large, the activity of peroxidase A6 represents only a fraction of the total activity of the crude extract.

3.2. Purity analysis and determination of molecular weight

Fig. 4 shows the revelation of peroxidase A6 activity after native PAGE. The purified isoperoxidase shows a single band, only prolonged reaction time revealed negligible traces of activity below the band, suggesting that the isoperoxidase is highly free from other peroxidase isoenzymes.

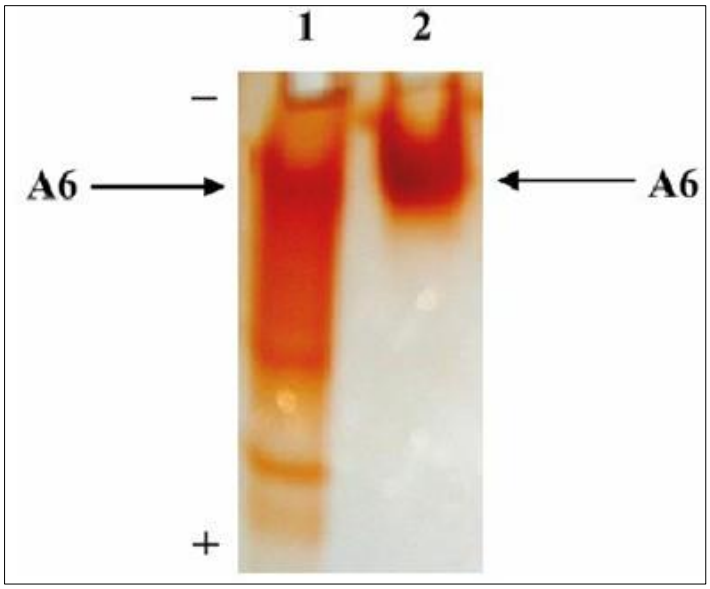

Figure 4 Native-PAGE of the partially purified anionic isoperoxidases. Lane 1: anodic peroxidases of the crude extract; lane 2: purified peroxidase A6

SDS-PAGE analysis of peroxidase A6 revealed a single band of 40.3 kDa using prestained molecular markers (New England Biolabs P7708S) or 42.6 kDa when using other non-prestained molecular markers (New England Biolabs P7702S) (Fig. 5). The RZ value (A_{403}/A_{280}) which represents the ratio of absorbance of the heme moiety to that of the apoprotein moiety was 2.4. The calculated RZ ratio is beside the range of values generally found for pure preparations of plant heme peroxidases [14, 15], which leads to the conclusion that peroxidase A6 is a classical plant peroxidase. The calculated molecular weight is also in the range of the given molecular weights for diverse other classical plant peroxidases [16, 17].

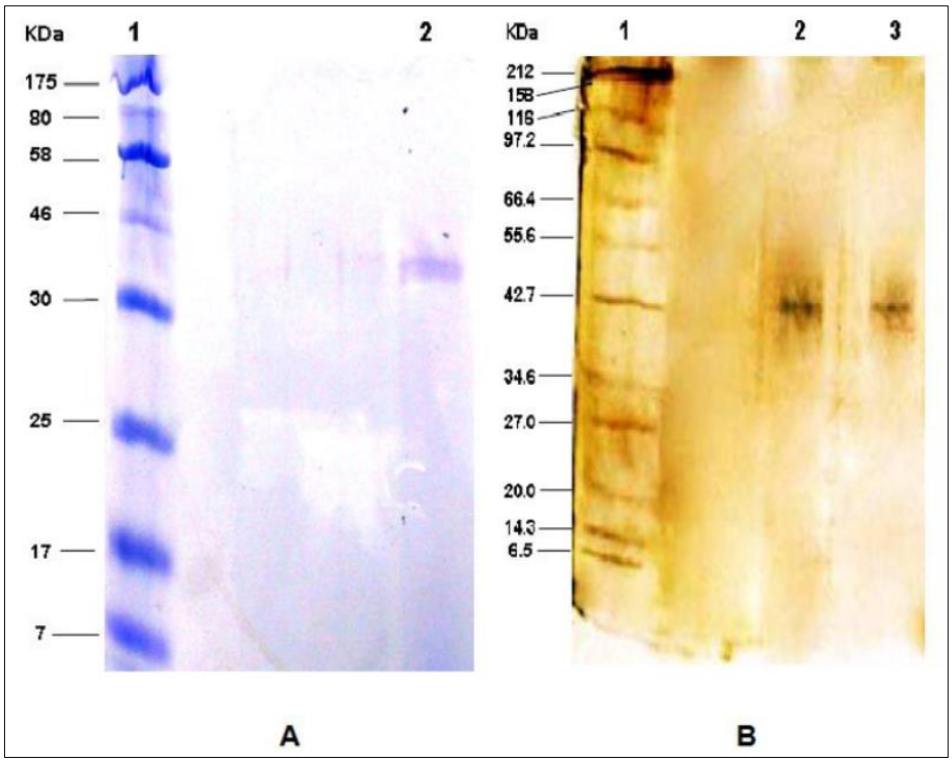

Figure 5 Purity analysis and determination of the molecular mass of isoperoxidase A6 by SDS-PAGE. A: Lane 1: molecular weight markers (prestained New England Biolabs P7708S); Lane 2: Purified peroxidase A6. The revelation of the bands was done by staining with Coomassie blue. 1.3 µg of peroxidase A6 and 50 µl of solution containing the markers were loaded, which corresponds to about 5 µg of each marker (the concentration of each marker is about 100 µg / ml according to the supplier New England Biolabs). B: Lane 1: molecular weight markers (non-prestained New England Biolabs P7702S); Lane 2: purified peroxidase A6, treated with β-mercaptoethanol. Lane 3: purified peroxidase A6, treated in the absence of β-mercaptoethanol. The revelation of the bands was made by silver nitrate staining. About 100 ng of peroxidase A6 and 10 µl of solution containing the markers were loaded

3.3. Optimal pH for diverse peroxidase substrates

Isoenzyme A6 is very active at acid pH. The pH effect on its activity for the various reducing substrates used in this study is represented in Fig. 6. Maximum activity is respectively at pH 3 for ABTS, pH 4 for OPD, and pH 6 for the others (TMB, DAB, and O-dianisidine).

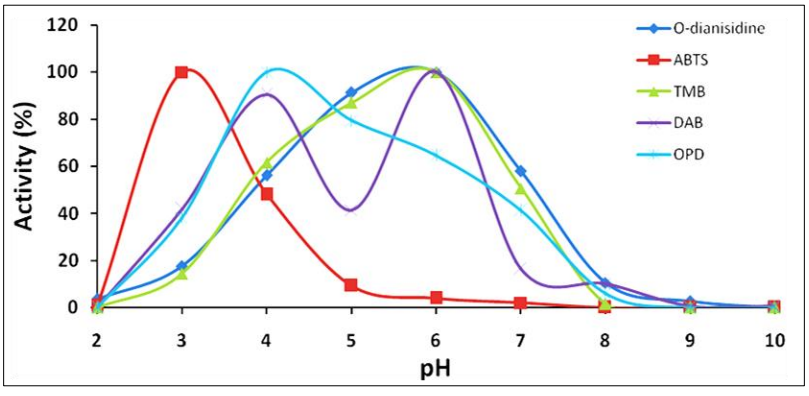

Figure 6 Effect of pH on peroxidase A6 oxidizing capacity towards various substrates. Each value is the mean value ± standard deviation of 3 measurements.

This preferential activity under acid conditions is also a characteristic of most peroxidases from other plants [6, 18]. However, the very acid optimal pH for ABTS oxidation is rare in plant peroxidases, only observed for the peroxidase of the African oil palm tree [19]. This suggests a great stability of peroxidase A6 in an acid environment.

3.4. Kinetic parameters for diverse peroxidase substrates

The apparent km and Vmax were obtained at the optimal pH of oxidation of each substrate, and those of H_2O_2 were estimated by using ABTS as reducing substrate, at the optimal pH of oxidation of this reducing substrate as obtained in this study (Table 2).

Table 2 Kinetic parameters of peroxidase A6 for H_2O_2 and five reducing substrates

	O-Dianisidine	**ABTS**	**TMB**	**DAB**	**OPD**	**H_2O_2**
Km (mM)	3.50	0.12	1.81	0.05	17.22	2.53
Vmax (U.µg^{-1} protein)	4.27	6.12	6.83	0.22	129.87	107.53
Kcat/Km (min^{-1}. mM^{-1})	5.12×10^4	2.22×10^6	1.59×10^5	1.82×10^5	3.17×10^5	1.79×10^6

Activity measurements were done in duplicate.

The Km values obtained are comparable to those of other peroxidases known for their great sensitivity towards various substrates [20]. The values of catalytic efficiency Kcat/Km obtained with peroxidase A6 are very high, in particular those relating to the oxidation of ABTS and the reduction of H_2O_2. For example, we obtained a Kcat/Km ratio of 1.79×10^6 mM^{-1}.min^{-1} for the reduction of H_2O_2 by peroxidase A6, while it was found by others that this ratio is 2.58×10^3 mM^{-1}.min^{-1} (43 mM^{-1}.s^{-1}) for the peroxidase from *Ziziphus jujuba* fruit [21], 1.779×10^3 mM^{-1}.min^{-1} (29.66 mM^{-1}.s^{-1}) for the peroxidase from *Coleus Forskohlii* [6]. The calculated values of catalytic efficiency revealed that the reducing substrates can be classified by decreasing order of specificity for peroxidase A6 as follows: ABTS, OPD, DAB, TMB, O-dianisidine. However, this classification does not take into account the fact that the determination of these kinetic parameters was made according to the optimal pH for each of these substrates. According to the operational pH of a biotransformation process, the catalytic efficiency can be very different. For example, by comparing the activity of peroxidase A6 for ABTS at pH 3 and pH 6, it can be noted that this activity at pH 6 accounts for only 4 % of that obtained at the optimal pH (pH 3); in addition, the activity of peroxidase A6 concerning the oxidation of OPD at pH 6 accounts only for 67 % of that calculated at the optimal pH (pH 4). Moreover, considering that the affinity of the enzyme for the substrate depends also on the pH, that could also contribute to upset this classification if we compare the catalytic efficiencies at the same pH.

3.5. Optimal temperature

Fig. 7 illustrates that the activity was best performed at a temperature of about 60 °C.

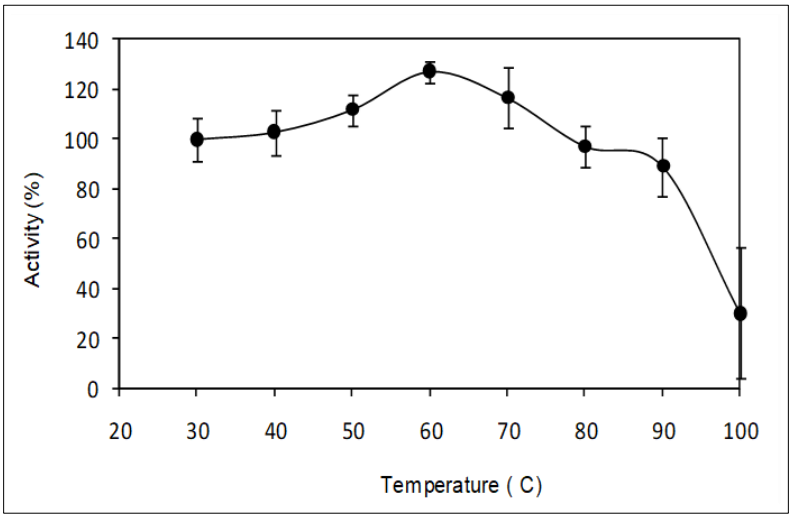

Figure 7 Effect of reaction temperature on peroxidase A6 activity. Each value is the mean value ± standard deviation of 3 measurements. Error bars indicate the standard deviation from the mean value

The optimal temperature of activity of peroxidase A6 is higher than those obtained for the major part of other peroxidases. Indeed, several peroxidases have their optimal temperature of activity ranging between 30 and 50 °C [4, 5, 6]. This property of peroxidase A6 is a great advantage on the practical level, for the rate of enzyme catalysis generally increases with an increase in temperature until a critical point known as the optimum temperature beyond which

denaturation of the enzyme is initiated and the reaction rate begins to decrease. Thus, a high optimum temperature is often associated with a high thermal stability of the enzyme.

3.6. Thermal stability

The thermostability of peroxidase A6 was determined by incubation at various temperatures and measurement of residual activities at intervals of incubation time, compared with that of control. The inactivation kinetics of the enzyme at pH 8 were determined for each temperature. They fitted a first-order reaction, and the half-lives of peroxidase A6 were 3.06 weeks, 13.5 hours, 15.5 min, and 3.5 min at 50 °C, 70 °C, 80 °C, and 90 °C respectively (Fig. 8).

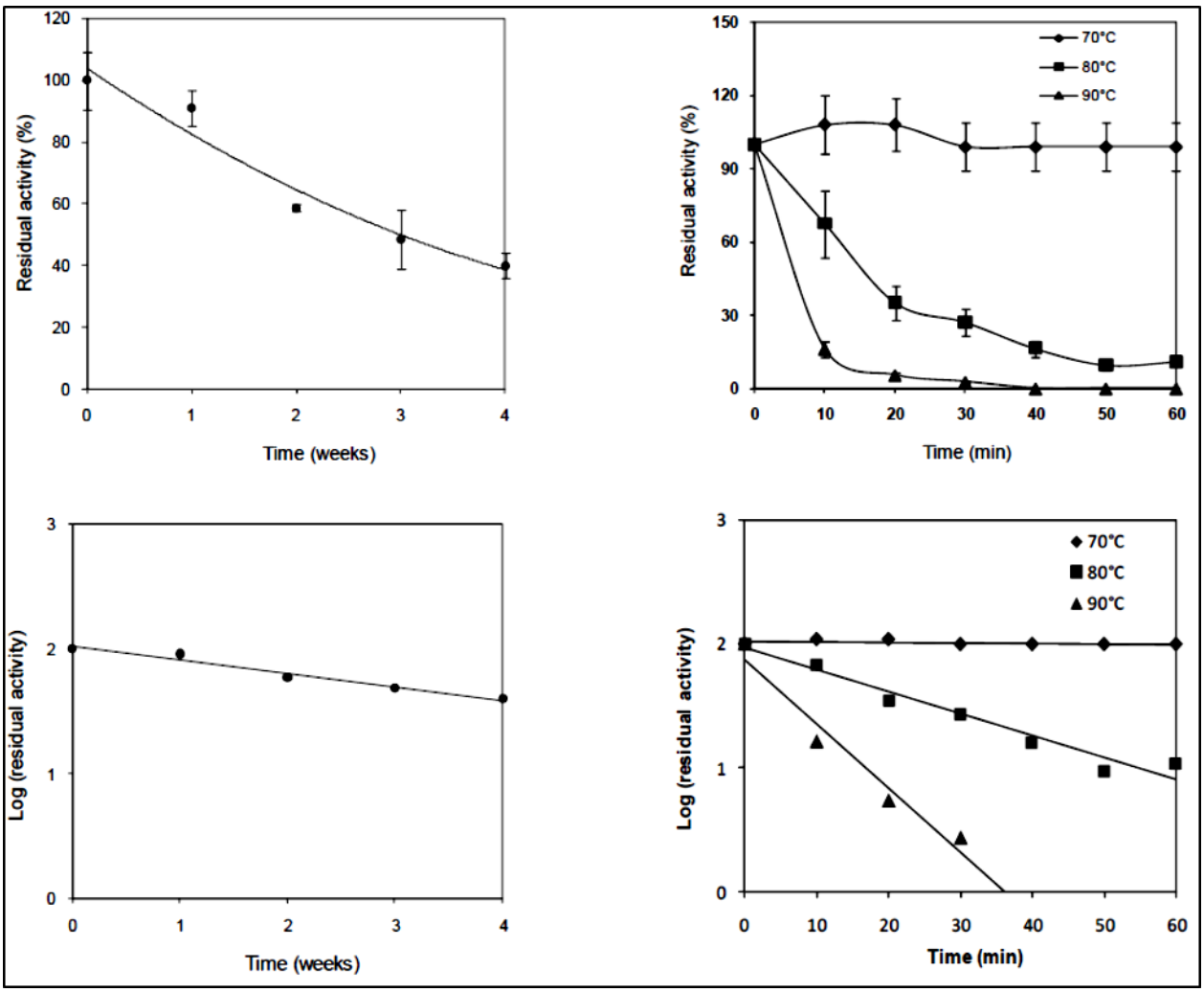

Figure 8 Thermal inactivation of peroxidase A6 activity at high temperatures. Each value is the mean value ± standard deviation of 3 independent experiments. Error bars indicate the standard deviation from the mean value. In the top: Residual activity at 50 °C (left); residual activity at 70 °C, 80 °C and 90 °C (right). In bottom: plots of Log (residual activity) at 50 °C (left) and Log (residual activity) at 70 °C, 80 °C and 90 °C (right)

These results testify to great stability of peroxidase A6 at high temperatures. The calculated half-lives at 70 °C, 80 °C, and 90 °C are largely higher than what is reported for other peroxidases. For example, maize cobs peroxidase loses activity after only 10 min at 60 °C [22], moreover, horseradish peroxidase, the most studied peroxidase, loses its activity only after 10 minutes of treatment at 70 °C at pH 7 [18]. Only palm tree and soybean peroxidases have been reported to have similar or greater stability among plant peroxidases [23]. In addition, at 50 °C, peroxidase A6 has an impressive half-life of 3.06 weeks. The great stability of peroxidase A6 peroxidase at fairly high temperatures could be an advantage for processes like hydrogen peroxide biosensing where peroxidases must catalyze reactions for several hours at 37 °C, and wastewater treatment which is held at temperatures up to 60 °C for hours [1]. Furthermore, nucleic acid-sensing by electrochemical processes which rely on denaturing paired nucleic acid strands at temperatures above 50 °C requires thermostable electrochemical devices, notably nucleic acid-thermostable peroxidase probe [24].

Thermal inactivation of peroxidase A6 at high temperatures followed first-order kinetics as plotting log (residual activities) vs. time gave straight lines. This suggests that the denaturation of peroxidase A6 can be interpreted by conformational changes between a native state and a final denatured state, which could be analyzed by the Arrhenius equation. The Arrhenius plot for calculating activation energy for heat inactivation of peroxidase A6 is shown in Fig. 9. The activation energy for the thermal inactivation reaction was determined to be 221.5 kJ/mol.

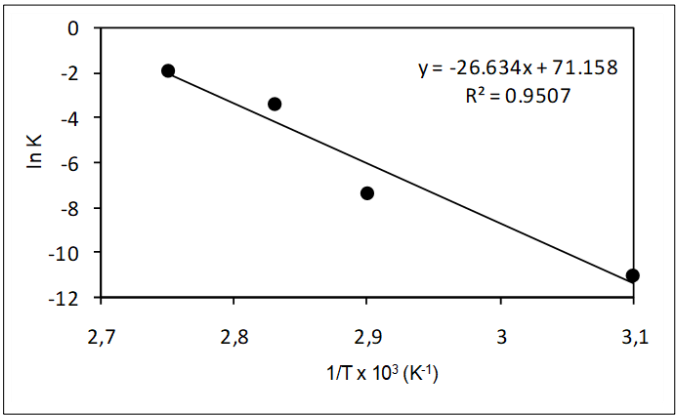

Figure 9 Arrhenius plot of thermal inactivation reaction of peroxidase A6 at pH 8

This value is significantly higher compared for example to that reported for cucumber peroxidase (127.99 KJ/mol) [25] and for *Ziziphus jujuba* peroxidase (120.9 KJ/mol) [21].

3.7. Storage stability at room temperature

We observed a very low inactivation rate of peroxidase A6 during its storage at room temperature for 6 months. In the first 4 months, the variation of the inactivation rate is linear, and at the end of the fourth month, the residual activity was still 94 %. In the 2 following months, a more notable reduction of activity was observed, the residual activity being then reduced respectively to 86.64 % and 76 % of the initial activity in the 5th and 6th month (Fig. 10).

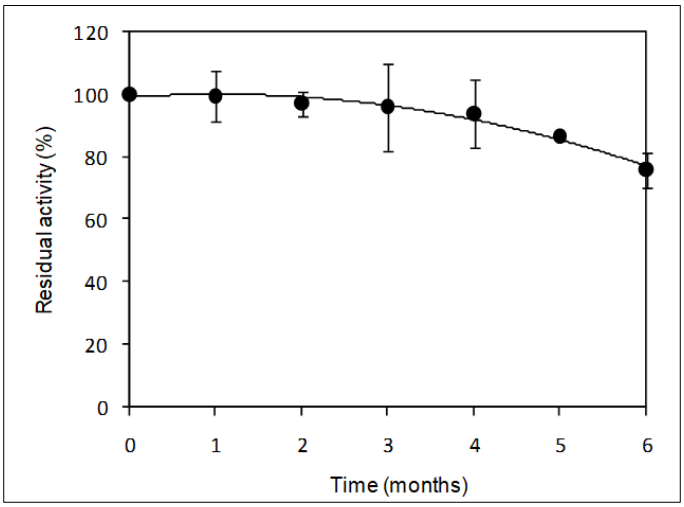

Figure 10 Inactivation of peroxidase A6 following exposure to room temperature. Each value is the mean value ± standard deviation of 3 measurements. Error bars indicate the standard deviation from the mean value

Scarce data affirm that horseradish peroxidase completely loses its activity after 4 months under similar conditions and that peroxidases from crude extracts of *Picea abies* L. Karst. needles lose up to 60 % of their activity only after 1 month of storage at 24 °C [26]. Other scattered data argue that soybean seed coat peroxidase conserves substantial activity after 1 year of storage under similar conditions. Indeed, compared to horseradish peroxidase, soybean peroxidase has, in addition to greater catalytic efficiency, a longer half-life at temperatures higher than the temperature of congelation, and so, it was found that soybean peroxidase is superior to horseradish peroxidase to help diagnose various viral, bacterial, and parasitic diseases, including AIDS and malaria. Thus, the stable characteristics of peroxidase A6 at ambient

temperature for months can make it possible to avoid the cycles of freezing/thawing of immunoconjugates used in techniques such as ELISA, which generally contributes to the denaturation of reagents.

3.8. Effect of metal ions

Magnesium salt had a significant effect on the activity of the enzyme at ambient temperature (Fig. 11). The Mann-Whitney test shows a significant difference between the activity of the control and that of the enzyme in the presence of magnesium salt (P = 0.043), showing that this salt produces an inhibiting effect. Some studies show that Mg^{2+} can behave like activator or inhibitor of peroxidases. For example, Glutathion peroxidase of *Chlamydomonas reinhardtii* is inhibited by weak concentrations (1mM) of Mg^{2+} [27] while the activity of peroxidase from *Luffa aegyptiaca* fruit is enhanced by Mg^{2+} [28].

On the other hand, the thermostability of peroxidase A6 is increased in the presence of calcium salt. After incubation at 80 °C for 10 min, the residual activity in the presence of this salt is 8 times higher than that of the enzyme alone. However, zinc and manganese salts reduced significantly the thermostability of the enzyme. The stabilizing effect of Ca^{2+} had already been observed with different plant peroxidases [29].

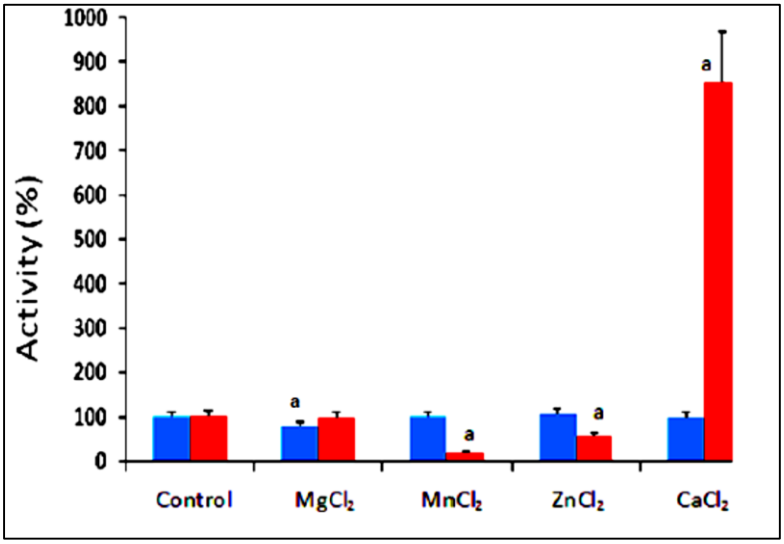

Figure 11 Effect of metal salts on the activity at room temperature (blue rectangles) and the thermal stability at 80 °C (red rectangles) of peroxidase A6. Each value is the mean value ± standard deviation of 4 independent experiments. Error bars indicate standard deviation from the mean value. "a", significantly different from the control (Mann-Whitney test, P < 0.05)

3.9. Effect of sodium azide

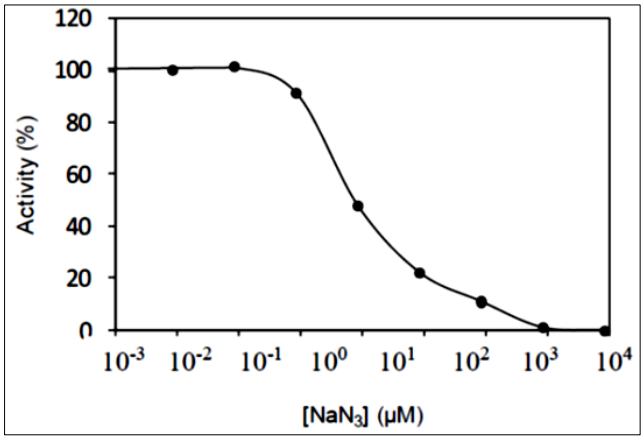

Figure 12 Inhibition of peroxidase A6 activity by sodium azide. Measurements were done in duplicate

Peroxidase A6 is significantly inactivated by sodium azide at concentrations as low as 1 µM. About 10 µM is the estimated IC_{50} value. Nevertheless, the enzyme's activity remained largely unaffected by concentrations less than 1 µM (Fig. 12).

While we found that very low IC_{50}, Almulaiky obtained a value of 1049 µM for peroxidase from *Coleus Forskohlii* [6]. Therefore, for applications of peroxidase A6 in antigen detection techniques, sodium azide must consequently be eliminated or reduced from the commercial preparations of antibodies used for conjugation with peroxidases. That inhibition was also observed for other peroxidases including horseradish peroxidase, and is thought to be due to the binding of the azidyl radical to the heme nucleus. This suggests that peroxidase A6's structure contains a heme group, indicating that it is a member of class III plant peroxidases.

4. Conclusion

The goal of this study was to isolate and characterize a peroxidase from Bambara groundnut, a vegetable that can withstand harsh environmental conditions, to assess its potential for biotechnology applications. After purification of this peroxidase that we had previously named peroxidase A6, a more detailed study of its activity was carried out by monitoring its catalysis of hydrogen peroxide reduction by five phenolic substrates among the most used in peroxidase applications. This isoperoxidase has a high catalytic efficiency, as we discovered. Additionally, it has a half-life of three weeks at 50 °C, maintained nearly all of its activity after an hour of incubation at 70 °C, and preserved over 95 % of its activity after four months of room temperature storage. This peroxidase is strongly stabilized by Ca^{2+} against heat treatments, which may enable it to expand its range of applications. Its high room temperature stability may enable a reduction in the freezing/thawing cycles, which often lead to the reagents becoming denaturized. From an economic perspective, this exceptional characteristic is helpful since it can reduce the cold chain of preservation, which is problematic in many ways, particularly in Africa.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

References

[1] Kyomuhimbo HD, Feleni U, Haneklaus NH, Brink H. Recent Advances in Applications of Oxidases and Peroxidases Polymer-Based Enzyme Biocatalysts in Sensing and Wastewater Treatment: A Review. Polymers. 2023; 15:3492. https://doi.org/10.3390/polym15163492

[2] Soy S, Sharma SR, Nigam VK. Bio-fabrication of thermozyme-based nano-biosensors: their components and present scenario. J Mater Sci: Mater Electron. 2022; 33:5523–5533

[3] Hussian CHAC, Leong WY. Thermostable enzyme research advances: a bibliometric analysis. J Genet Eng Biotechnol. 2023; 21:37. https://doi.org/10.1186/s43141-023-00494-w

[4] Askari H, Rahimian A, Aminian M. Purification and Biochemical Characterization of Two Anionic Peroxidase Isoenzymes from Raphanus sativus L. var niger Roots. Appl Biochem Biotechnol. 2022; 194(5):2219-2235. doi: 10.1007/s12010-021-03736-0

[5] Saikia S, Gogoi RD, Yadav M, Yadav HS. Isolation, purification and characterization of peroxidase from Raphanus sativus and its applications in biotransformation of cresols. Biocatal Agric Biotechnol. 2022; 46:102540 https://doi.org/10.1016/j.bcab.2022.102540

[6] Almulaiky YQ. Peroxidase from Coleus Forskohlii: Purification and Biochemical Characterization. Int J Nutr. 2020; 5(1):9-20. https://doi.org/10.14302/issn.2379-7835.ijn-19-3139

[7] Zhanga R, Hea L, Shena J, Miaoa Y, Tanga X, Wua Q, Zhoua J, Huang Z. Improving low-temperature activity and thermostability of exo-inulinase InuAGN25 on the basis of increasing rigidity of the terminus and flexibility of the catalytic domain. Bioengineered. 2020; 11(1): 1233–1244. https://doi.org/10.1080/21655979.2020.1837476

[8] Mbassi YMEL, Evehe MSB, Mbacham W, Muluh JP. Identification and partial purification of thermally stable peroxidase isoenzymes from seedlings of Vigna sp. (V) landrace Vn, World J Adv Res Rev. 2021; 12(03) : 476-483. https://doi.org/10.30574/wjarr.2021.12.3.0706

[9] Mbassi YMEL, Evehe MSB, Mbacham W, Muluh JP. Heat stable peroxidases from Vigna sp (V). African J Biotechnol. 2011; 10:3168-3175. https://doi.org/10.5897/AJB09.1720

[10] Bradford MM. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal Biochem. 1976; 72: 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

[11] McLellan T. Electrophoresis buffer for polyacrylamide gels at various pH. Anal Biochem. 1982; 126:94-99. https://doi.org/10.1016/0003-2697(82)90113-0

[12] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680-685. https://doi.org/10.1038/227680a0

[13] Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc. 1934; 56: 658-666. https://doi.org/10.1021/ja01318a036

[14] Huddy SM, Hitzeroth II, Meyers AE, Weber B, Rybicki EP. Transient expression and purification of horseradish peroxidase in Nicotiana benthamiana. Int J Mol Sci. 2018; 19 (115) 13. https://doi.org/10.3390/ijms19010115

[15] Kwak SS, Kim SK, Lee MS, Jung KH, Park IH, Liu JR. Acidic peroxidases from suspension cultures of sweet potato. Phytochemistry. 1995; 39:981-984. https://doi.org/10.1016/0031-9422(95)00098-R

[16] Abdel-Aty AM, Salama WH, El-Badry MO, Salah HA, Barakat AZ, Fahmy AS, Mohamed SA. Purification and characterization of peroxidases from garden cress sprouts and their roles in lignification and removal of phenol and p-chlorophenol. J Food Biochem. 2021; 45(1) e13526. https://doi.org/10.1111/jfbc.13526

[17] Zeyadi M. Purification and characterization of peroxidase from date palm cv. Agwa fruits. International Journal of Food Properties. 2019; 22(1):1910-1919. https://doi.org/10.1080/10942912.2019.1691589

[18] Navid M, Ghourchian H, Amininasaby M. Structural Stabilization and Functional Improvement of Horseradish Peroxidase upon modification of Accessible Lysines: Experiments and simulation. Biophys J. 2007; 92:1192-1203. https://doi.org/10.1529/biophysj.106.092858

[19] Sakharov IY, Blanco MKV, Sakharova IV. Substrate Specificity of African Oil Palm Tree Peroxidase. Biochemistry. 2002; 67 (9):1043-1047. https://doi.org/10.1023/A:1020534321683

[20] Conroy JM, Borzelleca DC, McDonell LA. Homology of Plant Peroxidases An immunochemical approach. Plant Physiol. 1982; 69:28-31. https://doi.org/10.1104/pp.69.1.28

[21] Zeyadi M, Almulaiky YQ. A novel peroxidase from Ziziphus jujuba fruit: purifcation, thermodynamics and biochemical characterization properties. Sci Rep. 2020; 10:8007 | https://doi.org/10.1038/s41598-020-64599-9

[22] Hathama Razooki H, Ali Waleed AA. An economical source for peroxidase: maize cobs. Karbala International Journal of Modern Science. 2022; 8 (2):article 11. https://doi.org/10.33640/2405-609X.3227

[23] Sakharov IY. Palm Tree Peroxidases. Biochemistry. 2004; 69(8):823-829. https://doi.org/10.1023/B:BIRY.0000040213.91951.bc

[24] van Gijlswijk RPM, Talman EG, Peekel I, Bloem J, van Velzen MA, Heetebrij RJ, Tanke HJ. Use of Horseradish Peroxidase- and Fluorescein-modified Cisplatin Derivatives for Simultaneous Labeling of Nucleic Acids and Proteins. Clin Chem. 2002; 48:1352-1359. https://doi.org/10.1093/clinchem/48.8.1352

[25] Chukwunyere IC, Ifeanyi UF, Amaechi OL, Amuche NJ, Ejike OK, Eluka CP, Joseph A, Nwamaka OM, Elijah JP. Evaluation of Inactivation Kinetics and Thermodynamic Properties of Peroxidase from Cucumis sativus for Biotechnological and Industrial Applications. J Adv Biol Biotechnol. 2021; 24(6):46–62. https://doi.org/10.9734/jabb/2021/v24i630221

[26] Has-Schön E, Lepeduš H, Jerabek L, Cesar V. Influence of Storage Temperature on Total Peroxidase Activity in Crude Extracts from Picea abies L. Karst. Needles. Croat Chem Acta. 2005; 78(3):349-353.

[27] Shigeoka S, Takeda T, Hanaoka T. Characterization and immunological properties of selenium-containing glutathione peroxidase induced by selenite in Chlamydomonas reinhardtii. Biochem J. 1991; 275:623-627. https://doi.org/10.1042/bj2750623

[28] Basumatary D, Yadav M, Nath P, Yadav HS. Catalytic Biotransformations and Inhibition Study of Peroxidase from Luffa aegyptiaca. Curr Organocatal. 2020; 7(2):149-157 DOI: https://doi.org/10.2174/2213337207666200211095038

[29] Plieth C, Vollbehr S. Calcium promotes activity and confers heat stability on plant peroxidases. Plant Signaling & Behavior. 2012 ; 7(6):650-660. http://dx.doi.org/10.4161/psb20065