Purification and partial characterization of a thermostable peroxidase isoenzyme from *Vigna* sp seedlings

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Abstract

A peroxidase isoenzyme (named A6 in a previous study) was purified from radicles of a *Vigna* species by a combination of gel filtration on Sephadex G-100, heat treatment, CM-cellulose chromatography and DEAE-cellulose chromatography. It has been successfully separated from other anionic isoperoxidases expressed in the same tissue. It has a molecular weight of about 41 kDa and exhibits a great activity toward the oxidation of O-dianisidine, ABTS, TMB, DAB and OPD at optimum pH (pH 3 for ABTS, pH 4 for OPD and pH 6 for the others) and toward the reduction of H_2O_2. Its very acid optimum pH for the oxidation of ABTS is not a characteristic of other peroxidases except African oil palm tree peroxidase. Apparent Km values for these substrates were respectively 3.50 mM, 0.12 mM, 1.81 mM, 0.05 mM, 17.22 mM and 2.53 mM; catalytic efficiencies were 5.12×10^4 $mM^{-1}.min^{-1}$, 2.22×10^6 $mM^{-1}.min^{-1}$, 1.59×10^5 $mM^{-1}.min^{-1}$, 1.82×10^5 $mM^{-1}.min^{-1}$, 3.17×10^5 $mM^{-1}.min^{-1}$and 1.79×10^6 $mM^{-1}.min^{-1}$. It has an optimum temperature of activity around 60°C, and its heat inactivation fit to the first-order kinetics, with half-lives of 3.06 weeks, 13.5 hours, 15 min and 3.5 min at 50°C, 70°C, 80°C and 90°C respectively. The calculated activation energy (E) for its thermal inactivation was found to be 221.5 KJ/mol at pH8. This peroxidase isoenzyme is stable for 4 months at room temperature, loosing only 5% of its initial activity over this period. Mg^{2+} inhibits the activity of the enzyme. The Ca^{2+} ions greatly increase the stability of this peroxidase at 80°C, while Mn^{2+} and Zn^{2+} reduce it. The enzyme is inhibited by sodium azide at concentrations above 1 µM with an IC_{50} value around 10 µM. This inhibition, in addition to the RZ value (A_{403nm}/A_{280nm}) evaluated at 2.4 confirms the presence at the active site of the enzyme of a heme group common to class III peroxidases. The unusual catalytic and thermal characteristics of A6 could make it a potent tool in several biotechnological applications, especially as part of kit for enzyme immunoassays and clinical diagnosis.

Introduction

Because of their broad distribution within the living world and the great diversity of their substrates, peroxidases are highly exploited in biotechnology. Considerable applications of peroxidases imply reactions proceeding at moderately high temperatures. It is the case in hydrogen peroxide biosensors where peroxidases must function for hours around 37°C (Heller and Vreeke, 1999), or for the wastewater treatment which proceeds at temperatures up to 60°C for hours (Pokora and Johnson, 1993). An extremely valid advantage to undertake biotransformations at high temperatures is the reduction of the risk of contamination by common mesophiles. High temperatures also increase reaction speeds because of a reduction in the viscosity and an increase in the diffusion coefficient of the substrates, which have a benefic influence on yields of production (Mozhaev, 1993; Krahe et al., 1996; Becker, 1997; Kumar and Swati, 2001).

However at high temperatures, most enzymes are destabilized, and lose completely or partially their catalytic potential. Although the majority of already studied peroxidases have an optimal activity between 30 and 50°C (Kim and Shoda, 1999; Marzouki et al., 2005; Alokail and Ismael, 2005; Sisecioglu et al.,

2010), only a few number can stand their exposure for hours to those temperatures. In addition, at the present time the major commercially available peroxidase is horseradish peroxidase, whose thermal stability is relatively weak (Purev et al., 2014 ; Wang et al., 2016). So, there is a need to find peroxidases with higher stability and different substrate specificity. This would obviously promote the development of new analytical methods and industrial processes and improve immunoenzymatic analytical kits where peroxidases are used as immunoconjugates.

However, an inherent problem with thermostable enzymes is that they have in general a low flexibility at ordinary temperature, and acquire all their catalytic potential only at temperatures higher than 50°C or 70°C (Dong and Zeikus, 1997). This is a major disadvantage for applications such as enzyme immunoassays which are done at ambient temperature. Useful thermostable enzymes for these diagnostic applications are those which have also a great activity at moderate temperatures, conditions compatible with the biological activity and the stability of the other reagents implied in the analysis.

Thermostable peroxidase isoenzymes were recently detected in the seedlings of *Vigna sp* (Mbassi and al, unpublished). Following their exposure to 80°C and 85°C for 10 min, these peroxidases still presented a substantial activity. One of these isoperoxidases had moreover the advantage of having a great activity in these seedlings compared to the others. Its isolation from this plant is thus necessary for a thorough study of its biotechnological potential, especially its kinetic towards diverse peroxidase substrates commonly used in immunoassays and its behaviour with respect to various heat treatments. The present study relates to the purification, study of substrate specificity and thermal stability of that peroxidase isoenzyme named A6 in earlier work (Mbassi et al., unpublished).

Material And Methods

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), 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.

Plant material:

Seeds of a *Vigna sp* 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 Yaounde.

Germination procedure:

Seeds were germinated according to the same procedure previously described (Mbassi et al., 2011). Seeds were sterilised with 10% sodium hypochlorite (v/v) for 20 min and rinsed 4 times with distilled water (for each washing, the water volume was such that its level is two fold that occupied by the seeds in the washing container). Seeds were spread out in Petri dishes (15 seeds per dish) over a layer of moistened filter paper. The latter was kept moist every 3 days with distilled water and maintained in dark at room temperature. After 9 days, seedlings were collected and then stored at -20°C, until further used.

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 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 min at room temperature under the above conditions.

Protein determination:

The protein concentration at the various stages of purification was determined by the Bradford method (1976) with bovine serum albumin as a standard.

Purification procedure:

Roots of 9-days old a *Vigna* species seedlings were ground with a mortar and pestle in 50mM ice-cold phosphate buffer pH7 containing 5mM EDTA, 1mM EGTA and 1mM 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 15000g for 20 minutes. The supernatant was mixed with

1.5 volume of ice-cold acetone and kept at -20°C overnigth. 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, 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 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 collection of active ones. The active fractions were pooled by peaks of activity, then, in order to identify the corresponding isoenzymes, the various groups were subjected to a native electrophoresis on polyacrylamide gel in the 2 directions of migration. A slow migrating anodic isoperoxidase (A6) was equilibrated in 10 mM Tris buffer pH 8 by loading on Sephadex G-25 previously washed with the same buffer. The actives 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 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.

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 (1982). Electrophoresis was carried out toward anode to separate anionic isoperoxidases and toward cathode to separate cationic isoperoxidases. The gel was run at a constant voltage of 200 V until the front dye reaches 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 (1970) 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 6X 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 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 reaches the resolving gel, then the voltage was increased to 200 V until the bromophenol blue reaches the lower end of the resolving gel. The revelation of bands was done by the Coomassie blue staining and the silver nitrate methods.

Substrate specificity:

Determination of optimum pH for catalysis of diverses 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 cuve. 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}).

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 for 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 to 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 (1934) was used to obtain the apparent Km and Vmax.

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 to incubate 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 cuve containing 5 µl of 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.

Thermal stability assays:

Effect of heat treatments

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 taken 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 ratio of activity of the test sample to that of the control which is an extract not heated. In parallel, 100µl of enzyme were incubated during 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. Activation energy (E) for thermal denaturation was calculated from the slope (-E/R) of Arrhenius plot, whose equation is as follows:

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

In this equation, k is a first-order kinetic constant that changes with temperature; E is referred by Arrhenius as representing the energy difference between the reactants and an activated species. The term E is therefore called 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 surmount in order to react. Therefore, high activation energy of a thermal denaturation reaction is a characteristic of most thermostable enzymes.

Storage stability at room temperature

One milliliter of purified enzyme was incubated in a narrowly closed tube for 6 months at room temperature (2326°C on average). Five microliters aliquots were taken each month and directly used for test activity. The reaction medium initially consisted of 5 µl of enzyme extract, 100 µl of 1 mM O-dianisidine, 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 ratio of activity of the test sample to that of the control which is an extract stored at-20°C.

Effect of metal ions on the activity and thermal stability of A6:

We tested the effect of some metal salts ($MgCl_2$, $MnCl_2$, $ZnCl_2$, and $CaCl_2$) on the activity and the thermostability of the isoperoxydase which we purified in this study. Five microliters of enzyme were incubated in presence and absence of 100 µl of a 1M 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 at 80°C during 10 minutes. The tube content was then transferred in a spectrophotometer cuve 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 that 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.

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 together the ideal characteristics for an application in clinical diagnosis and immunoassays, we evaluated the effect of sodium azide on the activity of the purified

peroxidase, since the commercial preparations of antibody used for the coupling of enzymes often contain sodium azide as preservative of microbial contamination. In addition, the inhibition of the activity of a peroxidase 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 aqueous solution of this compound were incubated with 5 µl of enzyme for about 30 minutes in a spectrophotometer cuve, 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 described previously.

Moreover, in order to verify the mechanism of inhibition, the activity of this isoenzyme was evaluated by varying the concentration of O-dianisidine (from 0.2 mM to 1 mM), at different fixed concentrations of sodium azide. The concentration of H_2O_2 was kept saturating (6 mM) in all cases. Activity measurements were done as described previously.

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.

Results

Isolation of A6:

The purification scheme of A6 is presented in table I. After precipitation of proteins by acetone, they were submitted to a 10 min heat treatment at 55°C in order to eliminate any contaminating proteins of low thermal stability, and the results show a very slight increase in the specific activity. 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 *Vigna sp* seedlings have nearby molecular weights. Following their separation by ion exchange chromatography on CM-cellulose, a group of close peaks between 2 broad peaks of activity were obtained (fig. 2).

Table I : Purification of 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 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 A6, which was eluted by 100 mM acetate buffer; the last peak represented the cationic peroxidases, eluted by 1 M acetate buffer. The fractions of the major peak of the group of close peaks was pooled and subjected to an ion exchange chromatography on DEAE-cellulose, in order 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). Fractions 105 to 111 were pooled.

Purity analysis and determination of molecular weight:

Figure 4 shows the revelation of 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.

SDS-PAGE analysis of A6 revealed a single band of 40.3 kDa using prestained molecular markers (New england Biolabs P7708S) or 42,6 kDa with reference to other non-prestained molecular markers (New england Biolabs P7702S). In addition, the bands obtained in the absence and in the presence of a reducing agent of disulfide bonds have an identical migration, which suggests that peroxidase A6 consists of a single polypeptide chain (figure 5). The determination of the molecular mass was made by extrapolation from the descriptive curve of the function LogM = f (Rf), where M represents the molecular markers mass and Rf their frontal ratios.

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.

Optimal pH for diverse peroxidase substrates:

Isoenzyme A6 is very active at acid pH. The effect of pH on its activity with respect to the various reducing substrates used in this study is represented in figure 6. One notes a maximum of activity respectively at pH 3 for ABTS, pH 4 for OPD, and pH 6 for the others (TMB, DAB and O-dianisidine).

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 II).

Table II: Kinetic parameters of A6 for H_2O_2 and five reducing substrates. Activity measurements were done in duplicate.

	O-Dianisidine	ABTS	TMB	DAB	OPD	H_2O_2
Km (mM)	3.50	0.12	1.81	0.05	17.22	2.53
Vmax ($U.\mu g^{-1}$ protein)	4.27	6.12	6.83	0.22	129.87	107.53
Kcat (min^{-1})	1.8×10^5	2.57×10^5	2.87×10^5	9.43×10^3	5.45×10^6	4.52×10^6
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

Optimal temperature:

The optimal temperature for the activity was around 60°C, as shown in figure 7.

Thermal stability:

The thermostability of A6 was determined by incubation at various temperatures and measurement of residual activities at intervals of incubation, comparing 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 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).

The Arrhenius plot for calculating activation energy for heat inactivation of A6 is shown in figure 9. The activation energy for thermal inactivation reaction was determined to be 221.5 kJ/mol.

Storage stability at room temperature :

We observed a very low inactivation rate of A6 during its storage at room temperature for 6 months. 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 %. 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 at the 5^{th} and 6^{th} month (fig 10).

Effect of metal ions :

The 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 produce an inhibiting effect. In the other hand, the thermostability of 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. In addition, zinc and manganese salts reduced significantly the thermostability of the enzyme.

Effect of sodium azide :

Sodium azide has considerably inactivated A6 starting from concentrations around 1 µM. Calculated IC_{50} value is approximately 10 µM. However, concentrations lower than 1 µM did not practically have an effect on the activity of the enzyme (fig. 12).

In order to determine the type of inhibition exerted by sodium azide on peroxidase A6, we measured the activity at different fixed concentrations of sodium azide, taking O-dianisidine as a variable substrate. The Lineweaver-Burk representation shows straight lines that intersect on the x-axis, indicating either non-competitive inhibition or irreversible inhibition (Figure 13).

Discussion

A peroxidase isoenzyme (named A6 in a previous study) has been successfully purified by the methods used in this study. Calculated RZ ratio (2.4) is besides in the range of values generally found for pure preparations of plant heme peroxidases, which leads to the conclusion that A6 is a classical plant peroxidase (Kwak et al., 1995 ; Padiglia et al., 1995 ; Christensen et al., 1998 ; Huddy et al., 2018). This purification however was achieved with poor yield. This poor yield is explained by the fact that seedlings of *Vigna sp* 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 A6 represents only a fraction of the total activity of the crude extract. The calculated molecular weight (40.3 KD) is also in the range of the given molecular weights for other classical plant peroxidases. Indeed, this molecular weight is similar to that of horseradish (44 kD) (Welinder, 1979), tobacco (37 kD) (Gazaryan and Lagrimini, 1996), sweet potato (37 kD) (Leon et *al*., 2002), peanut (37-40 kD) (Van Huystee et *al*., 2003) and soybean peroxidases (37 kD) (Gilliken and Graham, 1991).

The enzyme is very active at acid pH, since the optimal pH obtained for the oxidation of the 5 reducing substrates used in this study lie between 3 (for ABTS) and 6 (for O-dianisidine, DAB and OPD). This preferential activity under acid conditions is also a characteristic of the majority of peroxidases from other plants (Vitali et *al*., 1998; Marzouki et *al*., 2005; Yemenicioglu et *al*., 1999; Neptuno et *al*., 2003). However, the very acid optimal pH for ABTS oxidation was observed only for the peroxidase of the African oil palm tree (Sakharov et al., 2002), horseradish peroxidase for example showing an optimum of activity with respect to this substrate at higher pH (Childs and Bardsley, 1975). This suggests a great stability of A6 in an acid environment. A6 shows moreover a great sensitivity with respect to the reducing substrates used in this study and to hydrogen peroxide. The values of apparent Michaelis-Menten constant (Km) obtained are comparable to those of other peroxidases known for their great sensitivity towards various substrates. For example, Km for O-dianisidine and H_2O_2 were calculated to be 3.5 mM and 2.53 mM for A6 at pH 6 and pH 3 respectively, values which are similar to those of horseradish peroxidase isoenzyme VII (3.6 mM and 6.9 mM respectively) at pH 5.3 (Conroy et *al*., 1982). For an anionic peroxidase from *Brasssica napus* roots, Km values were found to be 0.37 mM, 0.84 mM and 1.4 mM respectively for O-dianisidine, ABTS and H_2O_2 (Agostini et *al*., 1999). The values of catalytic efficiency Kcat/Km obtained with 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 A6, while it was found by others that this ratio is $6x10^5$ and $2.28x10^5$ $mM^{-1}.min^{-1}$ respectively for the peroxidase from *Geotrichum candidum DEC 1* and horseradish peroxidase (Kim and Shoda, 1999). The calculated values of catalytic efficiency revealed that the reducing substrates can be classify by decreasing order of specificity for A6 as follows: ABTS, OPD, DAB, TMB, O-dianisidine. However, this classification is made without taking into account that the determination of these kinetic parameters was done at the beforehand given optimal pH for each of these substrates. According to the operational pH of a biotransformation process, the catalytic efficiency can be very different (Singh et al., 2008). For example, by comparing the activity of A6 with respect to 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 A6 with respect to 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 one compares the catalytic efficiencies at the same pH.

The optimal temperature of activity of A6 that we determined is higher than those obtained for the majority of other peroxidases. Indeed, several peroxidases have their optimal temperature of activity ranging between 30 and 50°C (Marzouki et *al*., 2005; Kim and Shoda, 1999; Alokail and Ismael, 2005; Sisecioglu et *al*., 2010; Altin et al., 2017). This property of A6 is a great advantage on the practical level, for the rate of enzyme catalysis generally increases with 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. The thermal stability of A6 was studied in detail, and the results obtained testify to a great stability with respect to the heat treatments. The calculated half-lives, 805 min, 15.5 min and 3.5 min respectively at 70°C, 80°C, and 90°C are largely higher than what is reported for other peroxidases. For example, sorghum peroxidase readily loses activity when incubated at temperature above 55 °C (Dicko et *al*., 2006), peroxidase of Garlic Allium sativum loses 50% of its activity in less than 20 min at 60°C (Marzouki et *al*., 2005), crude extracts of artichoke peroxidases loses almost all their activity after 10 min of incubation at 80°C (Sergio et *al*., 2007), and an anionic peroxidase from *Brassica napus* completely loses activity after 10 min of incubation at 70°C (Agostini et *al*., 1999). Moreover, horseradish peroxidase, the most studied peroxidase, loses its activity only after 10 minutes of treatment at 70°C at pH7 (Navid et al., 2007). Only palm tree and soybean peroxidases have been reported to have similar or greater stability among plant peroxidases (Sakharov, 2004; McEldoon and Dordick, 1996). In addition, at 50°C, A6 has an impressive half-life of 3.06 weeks. By comparison, peroxidase of Garlic *Allium sativum* loses 50% of activity only after 5 hours at 50°C, (Marzouki et *al*., 2005), peroxidases of Corn Root Plasma Membranes loses 40-50% of activity in 5 min (Mika and Lüthje, 2003). The great stability of A6 peroxidase at fairly high temperatures could be an advantage for processes like hydrogen peroxide biosensing where peroxidases must catalyse reactions for several hours at 37°C (Heller and Vreeke, 1999), and wastewater treatment which is held at temperatures up to 60°C for hours (Pokora and Johnson, 1993). Furthermore, nucleic acid-sensing by electrochemical processes which rely on denaturing paired nucleic acid strands at temperatures in excess of 50° C requires thermostable electrochemical devices, notably nucleic acid-thermostable peroxidase probe (Pokora and Johnson, 1993; Wolcott, 1992 ; van Gijlswijk et *al*., 2002). Thermal inactivation of A6 at high temperatures followed first-order kinetics as plotting log (residual activities) vs. time gave straight lines. This suggests that denaturation of A6 can be interpreted by conformational changes between a native state and a final denatured state, which could be analyzed by the Arrhenius equation. Thus, activation energy (221.5 KJ/mol) has been deduced from the Arrhenius plot. This value is significantly higher compared for example to that reported for horseradish peroxidase (159 kJ/mol) at pH 3, pH where it was shown that this enzyme is stable (Pina et *al*., 2001), to sorghum peroxidase (157 KJ/mol) at pH 5 (Dicko et *al*., 2006),

and to taro peroxidase (81.1 KJ/mol) (Yemenicioglu et *al*., 1999). In addition, regards to storage at ambient temperature, A6 lost only 5% of activity after 4 months at room temperature, and 24 % after 6 months. 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 (Has-Schön et al., 2005). Other scattered data argue that soybean seed coat peroxidase conserves a substantial activity after 1 year of storage under similar conditions. Indeed, compared to horseradish peroxidase, soybean peroxidase has, in addition to a greater catalytic efficiency, a longer half-life at temperatures higher than the temperature of congelation (Vierling et al., 2000), 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 A6 at ambient temperature during 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.

The study of the effect of metal salts on the activity and the thermal stability of A6 revealed that Mg^{2+} ions produce a slight inhibition at ambient temperature. Some studies show that metal salts can behave like activators or inhibitors of peroxidases. Horseradish peroxidase for example is activated by Ca^{2+} (Bakardjieva et al., 1999); glutathion peroxidase of Chlamydomonas reinhardtii is inhibited by weak concentrations (1mM) of Mg^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} (Shigeoka et al., 1991). With regard to the effect on thermal stability, the calcium salt behaved like a stabilizer, since the presence of $CaCl_2$ in the incubation medium has preserved a residual activity 8 times higher than that of control. The stabilizing effect of the Ca^{2+} ions had already been observed with horseradish peroxidase (Bakardjieva et al., 1999), and in moss (Christov et al., 2000).

However, sodium azide considerably inhibits the enzyme. So, for applications of A6 in techniques of antigen detection in clinical diagnosis or enzyme immunoassays, sodium azide must consequently be eliminated from the commercial preparations of antibody which are conjugated with peroxidases.

This inhibition has been shown to be irreversible in the case of horseradish peroxidase (Ortiz de Montellano et al., 1988), and would be due to the binding of the azidyl radical to the heme nucleus. Although other studies, carried out on other peroxidases, rather indicate a competitive type inhibition, or even a reversible inhibition (Liu et al., 2006), the graph that we obtained in our study would rather confirm the first hypothesis.

Declarations

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AKNOWLEDGEMENTS

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Figures

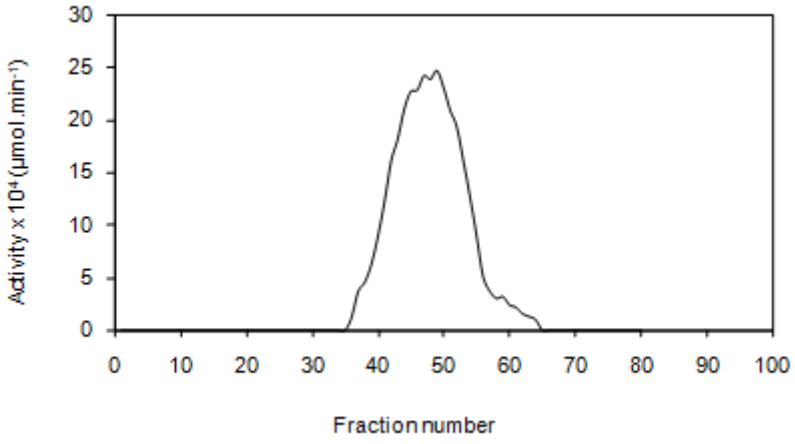

Figure 1

Elution profile after gel filtration on Sephadex G-100. Elution buffer was 0.1M phosphate buffer pH7.

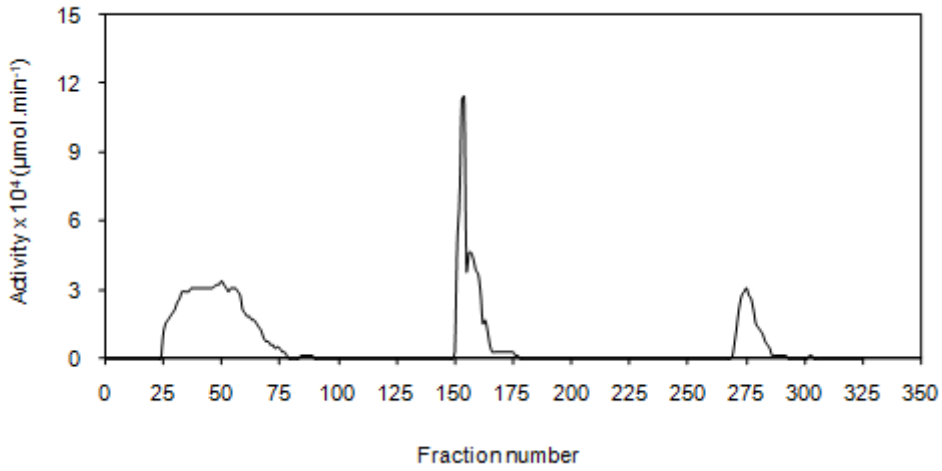

Figure 2

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

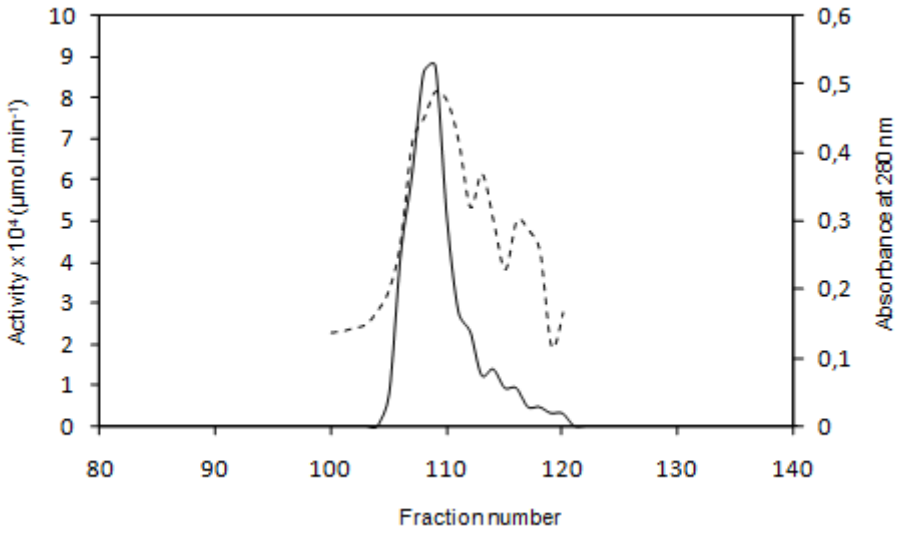

Figure 3

Elution profile of 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.

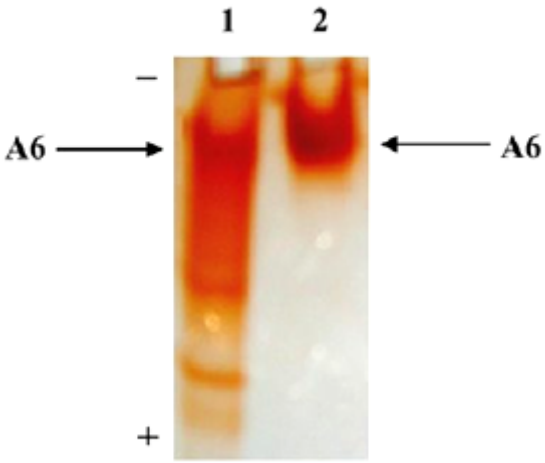

Figure 4

Native-PAGE of the purified anionic isoperoxidase A6. Lane 1: anodic peroxidases of crude extract ; lane 2: purified isoperoxidase A6.

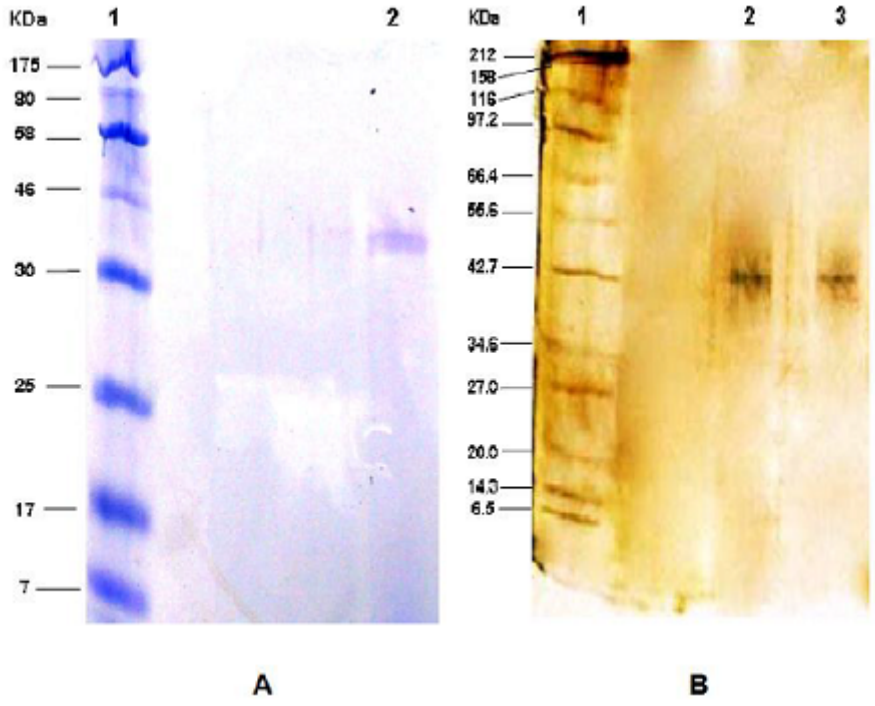

Figure 5

SDS-PAGE of purified A6 (detection with coomassie blue). Lane 1: molecular markers; lane 2: A6 after DEAE-cellulose chromatography

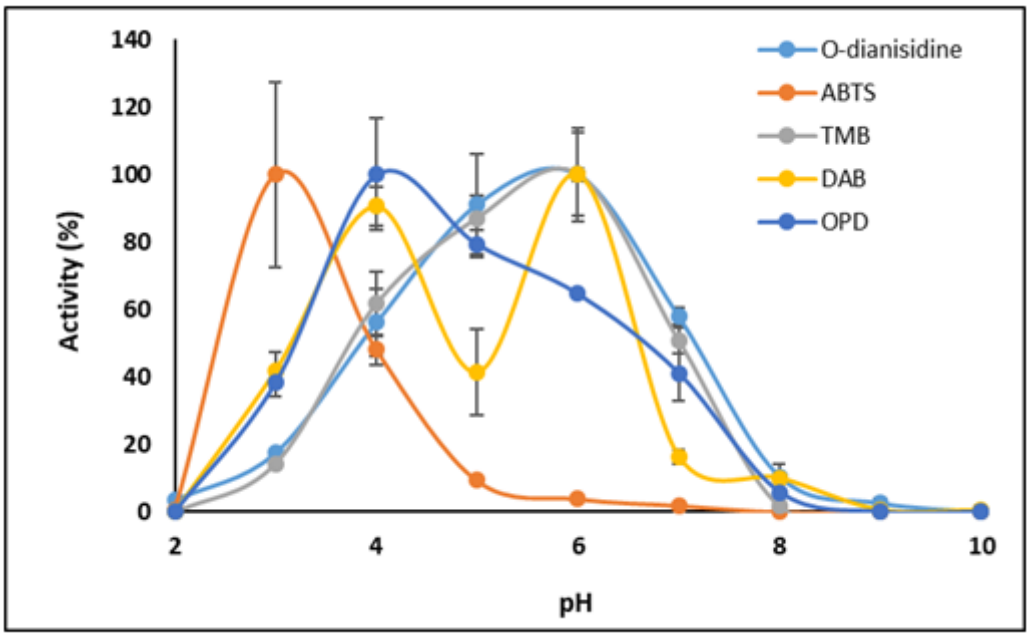

Figure 6

Effect of pH on A6 oxidising capacity towards various substrates. Each value is the mean value ± standard deviation of 3 measurements. Error bars indicate standard deviation from the mean value

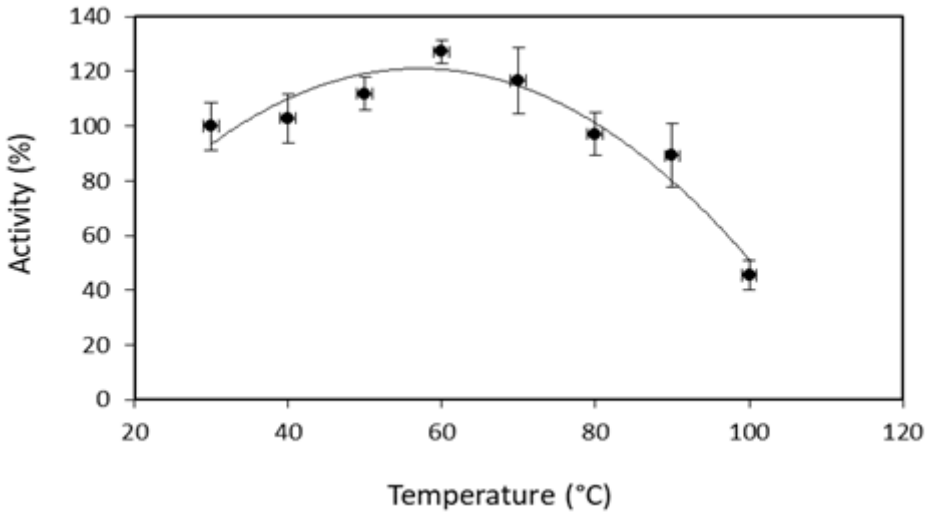

Figure 7

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

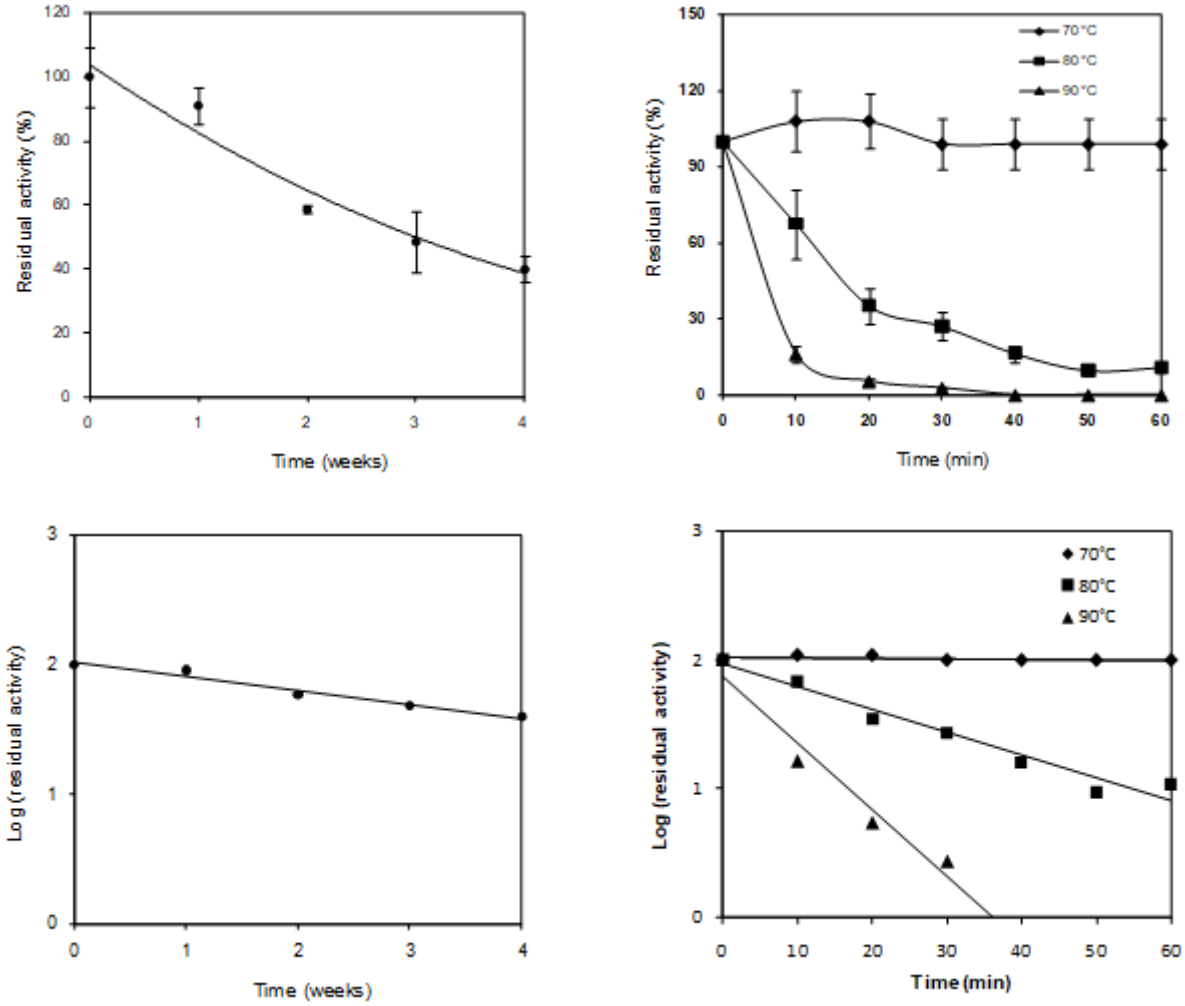

Figure 8

Thermal inactivation of A6 activity at high temperatures. Each value is the mean value ± standard deviation of 3 independent experiments. Error bars indicate 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).

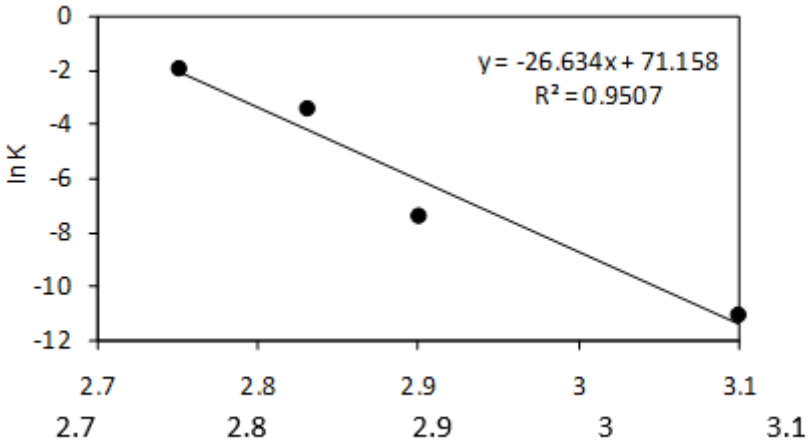

Figure 9

Arrhenius plot of thermal inactivation reaction of A6 at pH 8

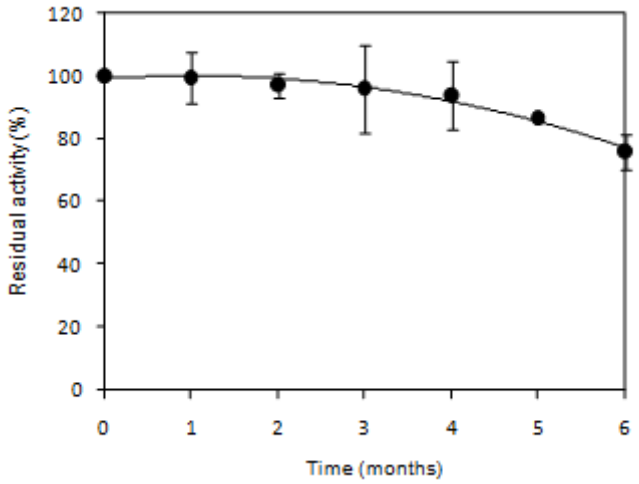

Figure 10

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

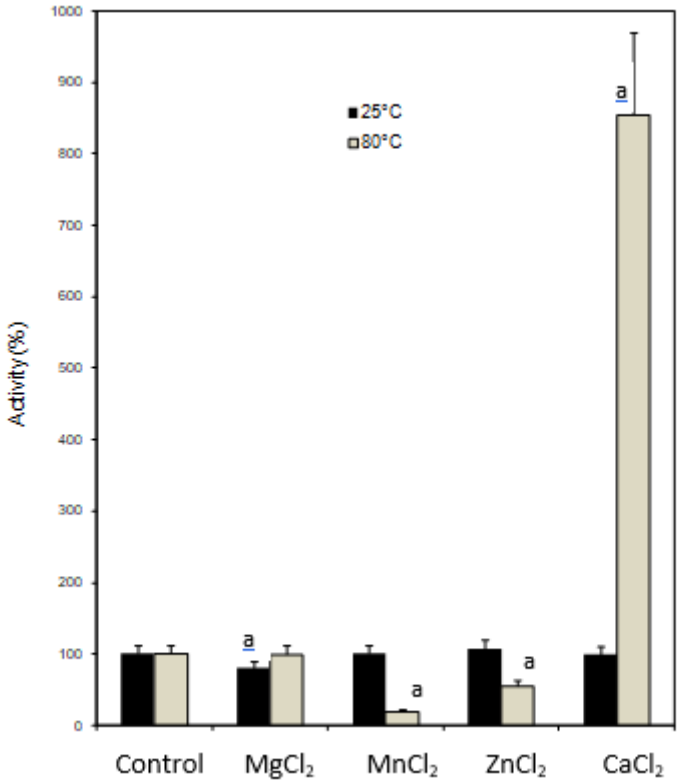

Figure 11

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

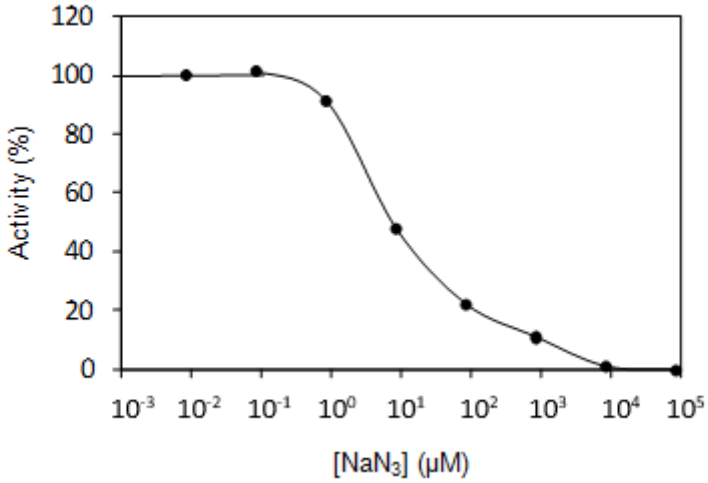

Figure 12

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

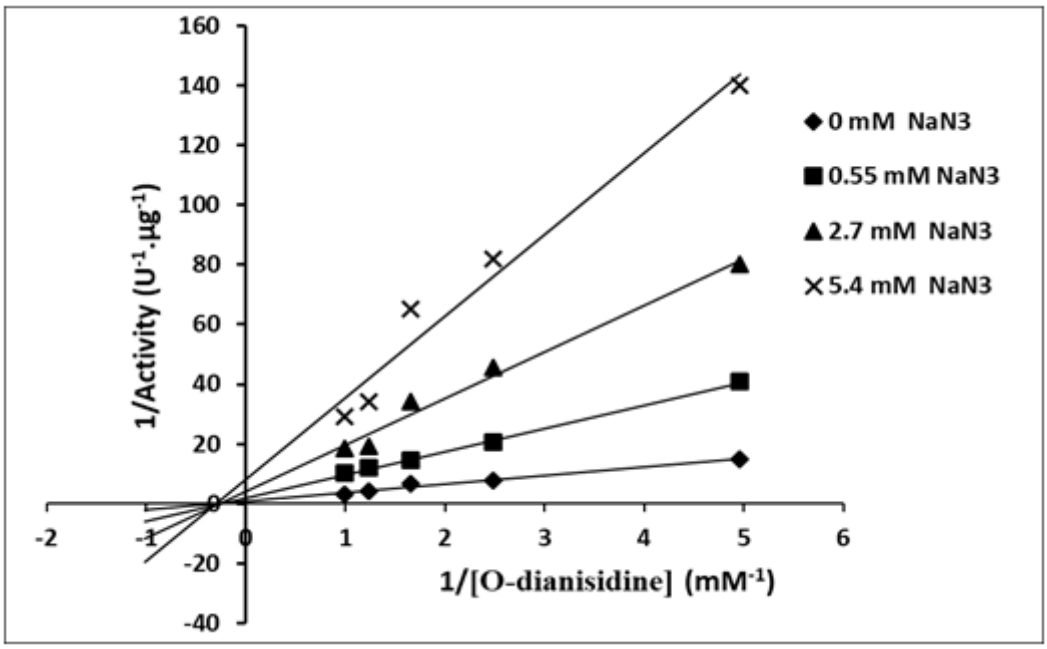

Figure 13

Lineweaver-Burk representation for A6 activity at varying concentrations of O-dianisidine, for different fixed concentrations of sodium azide.