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Identification and partial purification of thermally stable peroxidase isoenzymes from seedlings of *Vigna* sp. (V) landrace Vn

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Abstract

Several soluble peroxidase isoenzymes are expressed in a landrace of *Vigna* sp. cultivated in the north of Cameroon (landrace called Vn in previous study) during seed germination. There are at least two cathodic peroxidases and eight major anodic peroxidases as shown by their electrophoretic migration at pH 7.4 under native conditions. These isoperoxidases are more expressed in roots than in shoots. They have different thermal stability, so that heat inactivation kinetics of crude peroxidase extracts from roots do not fit the first-order model. The slow and intermediate migrating groups of anodic isoperoxidases retains a substantial activity after ten minutes of incubation at 80°C and 85°C. An anodic isoperoxidase (named A6 in this study) shows in addition to this great thermal stability, a high activity in seedlings and is expressed both in roots and shoots. The combination of those characteristics makes this isoperoxidase a potential candidate for biotechnological applications. Three major anodic isoperoxidases, of which A6 and another thermostable isoperoxidase, were successfully separated from each other by ion exchange chromatography on DEAE-cellulose, after precipitation of total proteins by ice-cold acetone. This offers the prospect of being able to characterize these isoperoxidases individually in future studies.

Keywords: Peroxidase; Vigna; Isoenzymes; Thermal stability; Purification

1. Introduction

Peroxidases are present in nearly the totality of living organisms, and are often found in the form of several isoenzymes, as the result of mutations occurred during evolution, and somewhat due to gene duplication. In plants, these isoperoxidases are expressed at different degrees according to the stage of growth or nature of the tissue [1, 2, 3]. These have various physiological roles in plant cells: they participate in different reactions including lignification, cross-linking of cell wall polysaccharides, and regulation of cell elongation, wound healing, phenol oxidation or oxidation of indol-3-acetic acid [4]. Many data pointed to the responsiveness of peroxidases to various biotic and abiotic stresses [5, 6, 7, 8]. While all these peroxidase isoenzymes have a same catalytic mechanism, they may differ markedly in their physicochemical properties, which are then a criterion of selection for biotechnological applications. One of these properties is their relative stability at high temperatures. Published data have shown a wide variation in heat inactivation characteristics of peroxidases, depending on the plant source of the enzyme, and among the isoenzymes present in the same plant [9, 10, 11, 12].

A previous work carried out in our laboratory showed a great thermal stability of crude extracts of soluble peroxidases from seedlings of *Vigna* sp., particularly a landrace cultivated in the hot northern part of Cameroon. That work revealed that the residual activity in this landrace was 67%, 34% and 3.4% after preincubation for 1 hour at 70°C, 75°C and 80°C respectively. For this same landrace, the peroxidase activity remained to more than 60% after preincubation for 3 weeks at 55°C and to 47% after storage during 1 year at room temperature [13].

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The present work aims at evaluating the number, tissue distribution and heat stability of isoperoxidases expressed during the germination of the above mentioned *Vigna* sp. landrace, and is exploring the possibility of isolating biotechnologically useful peroxidases from the plant in question.

2. Material and methods

2.1. Reagents

The following reagents were from SIGMA or SIGMA Aldrich: ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), O-dianisidine, PMSF (phenylmethylsulfonylfluoride), EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), monobasic and dibasic sodium phosphate, tris base, HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)), sodium hydroxide, diethylamino-ethyl cellulose, glycerol, ammonium persulfate, TEMED (tetramethylethylenediamine).

Some others were from MERCK (imidazole), FLUKA (acrylamide, bis-acrylamide), BAKER (bromophenol blue), QUIMICA DE MONTCADA SA (acetic acid), CARLO ERBA (37% Hydrogen chloride), and 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 *Vigna* sp. landrace with white seeds cultivated in the northern part of Cameroon were purchased at a local market of Yaounde.

2.3. Germination procedure

Seeds were germinated according to the same procedure previously described [13]. They were allowed to germinate in 4 petri dishes containing 15 seeds each. Samples of seedlings were collected separately after respectively 3, 6 and 9 days of germination. The fresh weight of each seedling was recorded. This biological material was stored at -20°C until use.

2.4. Protein extraction

Four gram of each sample was treated as previously described to extract proteins [13]. Furthermore, the crude extract was mixed with 1.5 volume of ice-cold acetone and kept at -20°C overnight to precipitate proteins. This mixture was thereafter 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. This extract was used for subsequent electrophoresis, chromatography and activity assays.

2.5. Activity assays

The enzyme activity was measured by using the reduction of hydrogen peroxide with either 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or O-dianisidine. Activity was measured by scanning through time points with a Biochrom Libra S12 spectrophotometer, following changes in absorbance of either product accrual: ABTS radical at 414 nm ($\varepsilon_{414\ nm}$= 31.1 mM^{-1} cm^{-1}) and O-dianisidine oxidation product at 460 nm ($\varepsilon_{460\ nm}$= 11.3 mM^{-1} cm^{-1}). Measurements were done at room temperature (~25°C).

2.6. 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 [14]. Each well contained 2 µl of extract. Electrophoresis was carried out toward the anode to separate anodic (neutral and anionic) isoperoxidases and toward the cathode to separate cathodic (cationic) isoperoxidases. The gels were run at a constant voltage of 200 V until the front dye (bromophenol blue for anodic peroxidases and methyl green for cathodic peroxidases) reaches the bottom.

Gels were stained by soaking for about 15 min into a mixture of 1 mM O-dianisidine and 0.5 mM H_2O_2 in acetate/acetic acid buffer pH 5.

2.7. Isoperoxidases thermal stability assays

Aliquots of 100 µl of peroxidase extracts from roots and shoots were preincubated in a microfuge tube for 10 min at 80°C, 85°C, 90°C and 95°C in a water bath. The heat treated extracts were immediately cooled in ice for at least 5 min, then supernatants were decanted by centrifugation at 15 000 g for 10 min at 4°C. Decanted supernatants were used as sources of peroxidases for gel electrophoresis. In another experiment, 100 µl of the supernatants were incubated in microfuge tubes for times varying from 1 min to 10 min at 80°C. Residual activity of these samples was then measured. Log (A /Ao) vs. time plots were established (Ao is the initial enzyme activity and A is the residual activity after heating for time t).

2.8. Purification procedure

Peroxidase crude extract was subjected to a gel filtration chromatography on Sephadex G-25 (4 cm × 1.5 cm) previously equilibrated with 10 mM Tris buffer pH 8. 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. 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 by using substrate systems O-dianisidine/H_2O_2 on one hand, and ABTS/H_2O_2 on the other hand. For each buffer concentration, elution was carried out until fractions with zero activity were obtained after collection of active ones. 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. The not well separated peroxidases were loaded again on a DEAE-cellulose column by groups of collected peaks, and stepwise elution was done with a wider range of buffer concentrations (50mM, 75 mM, 90 mM, 105 mM, 120 mM, 135 mM and 150 mM).

2.9. Statistical Analysis

Results are expressed as means ± standard deviations for the indicated number of experiments. Data were analyzed by one-way analysis of variance (ANOVA). When statistical differences of means were found, multiple comparisons were performed by the Fischer least significant difference test (LSD). *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Peroxidase activity during seed germination

Table 1 shows the evolution of the weight and peroxidase activity of the whole seedlings of *Vigna* sp. We noted that peroxidase activity increases relatively to the fresh weight of the seedling during germination. This increase is more important between the 3rd and the 6th day of germination.

Table 1 Variations with time of the fresh weight and peroxidase activity of *Vigna* sp. seedling during germination. $^{a, b, c}$: significantly different from activity at day 3, day 6 and day 9, respectively

	Day 3	**Day 6**	**Day 9**
Seedling fresh weight (mg)	44.23±1.75 ^{b,c}	198.2 ± 39.04 ^{a,c}	379.33 ± 50.63 ^{a,b}
Activity per mg of fresh seedling ($U.mg^{-1}$)	1.11±0.3 x 10^{-3} ^{b,c}	2.59±0.51 x 10^{-3} ^{a}	2.72±0.36 x 10^{-3} ^{a}

3.2. Tissue distribution of peroxidases isoenzymes in seedlings

To determine the tissue localization of peroxidase isoenzymes, we separated, using a scalpel, the roots and shoots of a 9-day-old sample of Vn landrace. Peroxidases were extracted from each part as described in "Materials and Methods" section. The electrophoresis implemented on the one hand in the cathode-anode direction, and on the other in the anode-cathode direction revealed at least 10 major isoenzymes, depending on their relative mobility, whose expression rates are different (Figure 1).

For anodic isoenzymes, we observe: two (named A6 and A5 in this study) slow migrating peroxidases (Rf = 0.1 and 0.15) from which one (A6) is expressed in both roots and shoots and A5 seems to be expressed only in roots; two (A4 and A3) intermediary migrating isoenzymes (Rf = 0.25 and 0.3) expressed in roots; one (A7) intermediary migrating isoenzyme (Rf = 0.23) expressed in shoots; two (A2 and A1) quick migrating isoenzymes (Rf = 0.48 and 0.5) expressed in roots and one (A8) quick migrating isoenzyme (Rf = 0.38) expressed in shoots (Figure 1A). Concerning cathodic peroxidases, there are two isoperoxidases (C2 and C1) expressed in both roots and shoots with low relative migrations. Moreover, their

expression rate seems to be similar in both parts, given the similarity in the intensities of the corresponding bands. The slowest, C2 (Rf = 0.08) is the most active. The fastest, C1 (Rf = 0.17) is less active (Figure 1B)

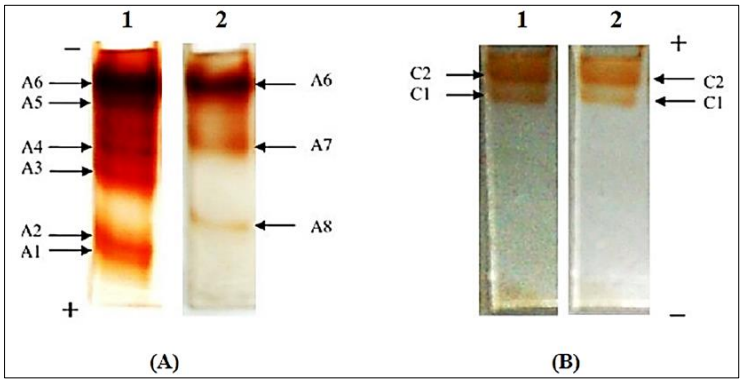

Figure 1 Distribution of peroxidase isoenzymes among roots and shoots of seedlings from *Vigna* sp. (A) Lane 1: anodic isoenzymes of roots; Lane 2: anodic isoenzymes of shoots; (B) Lane 1: cathodic isoenzymes of roots; Lane 2: cathodic isoenzymes of shoots

3.3. Thermal stability of the different isoenzymes

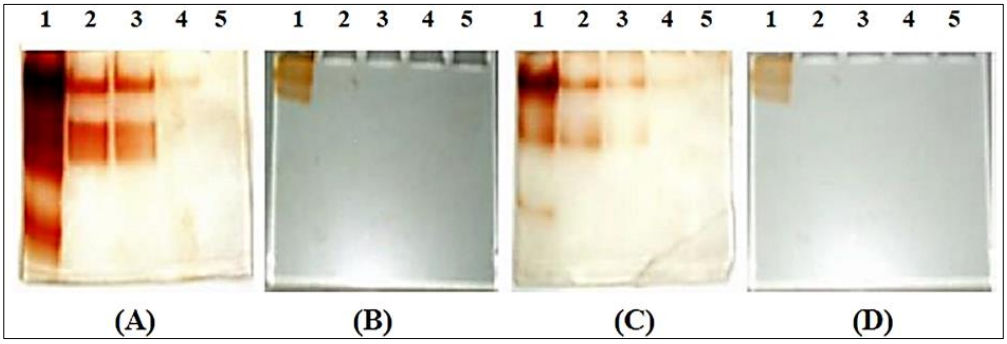

Figure 2 Thermal stability of soluble peroxidase isoenzymes from *Vigna* sp. expressed after 9 days of seedling growth. (A) anodic isoenzymes of roots; (B) cathodic isoenzymes of roots; (C) anodic isoenzymes of shoots; (D) cathodic isoenzymes of shoots. Lane 1: not heated extract; lane 2: heated at 80°C; lane 3: heated at 85°C; lane 4: heated at 90°C; lane 5: heated at 95°C

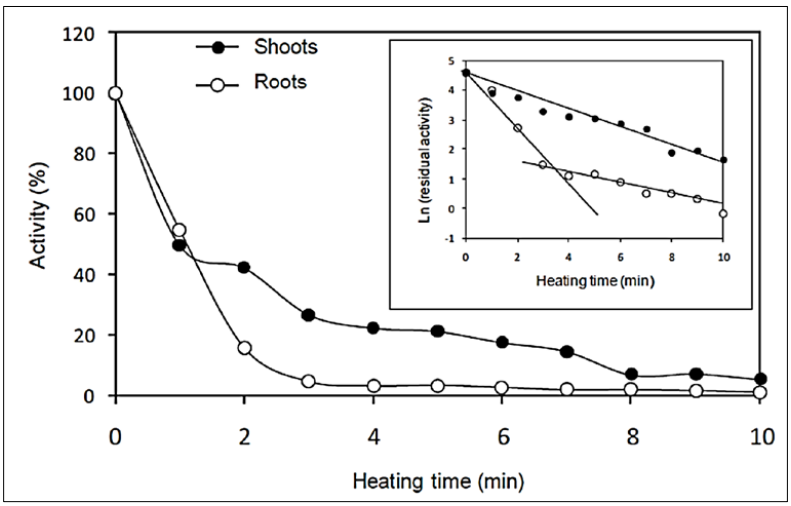

Figure 3 Kinetics of thermal inactivation (at 80°C) of crude extracts of soluble peroxidases of roots and shoots from *Vigna* sp. expressed after 9 days of seedling growth. Values are the means of 3 independent experiments. The standard error was less than 5 %

The results of heat stability experiments show that the slow and some intermediate migrating anodic isoperoxidases are relatively stable when heat treated for 10 min at 80°C and 85°C. After heating at 90°C for 10 min, the slow migrating isoenzyme A6 has yet a small activity. However, the fast migrating anodic isoenzymes, as well as all the cathodic isoenzymes are totally inactivated after heating for 10 min at any of those temperatures (Figure 2).

The inactivation kinetics of peroxidases of the roots (which contain a great quantity of isoperoxidases) gives a two-phase curve, while the kinetics obtained with the shoots (which contain mainly thermally stable anodic isoperoxidases) follows that of a first-order reaction (Figure 3).

3.4. Separation of shoots anodic isoperoxidases by ion exchange chromatography

Some anodic peroxidases showed greater thermostability in previous experiments. Peroxidase A6 in particular, which is found in both roots and shoots, showed the highest activity, in addition to its thermostability. Because zymograms revealed fewer isoperoxidases in the shoots compared to the roots, we felt that it would be easier to isolate peroxidase A6 from shoots for further study. Therefore, anodic peroxidases from shoots were separated by anion exchange chromatography, on DEAE-cellulose. Figure 4 shows that peroxidases A6, A7 and A8 were separated by this chromatography

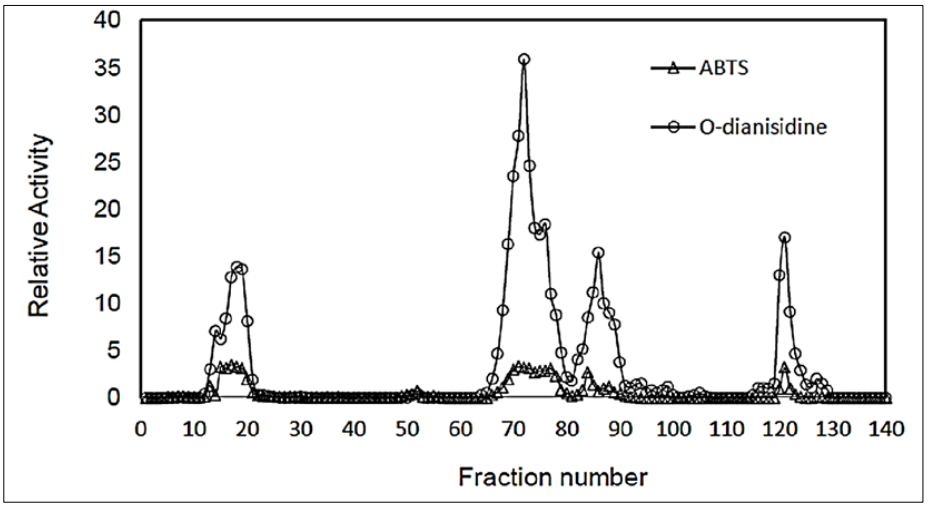

Figure 4 Elution profile of shoots peroxidases after ion exchange chromatography on DEAE-cellulose. Stepwise elution was carried out by increasing concentrations of tris/HCl buffer pH 8 (10 mM, 100 mM and 1 M)

Indeed, 4 groups of peaks appear on the figure. The composition of fractions corresponding to each peak was analyzed by native-PAGE (data not shown), and allowed the following observations: the first broad peak (fractions 13 to 23), eluted by washing the column, corresponded to a mixture of cationic isoperoxidases not retained by the matrix; fractions 65 to 80 corresponded to a mixture of at least two anionic isoperoxidases containing mainly A6, which were eluted by 100 mM tris/HCl buffer; fractions 81 to 93 corresponded to a mixture of at least three anionic isoperoxidases containing mainly A7, which was also eluted by 100 mM tris/HCl buffer; the last group of peaks (fractions 116 to 133) represented anionic isoperoxidases eluted by 1 M tris/HCl buffer.

Fractions 65 to 80 on one side and 81 to 93 on the other side were pooled and subjected separately to another ion exchange chromatography on the same matrix (Figure 5). However, for these new chromatography, elution was done successively with a greater number of buffer concentrations (50 mM, 75 mM, 90 mM, 105 mM, 120 mM, 135 mM and 150 mM), in order to eliminate any trace from minor peroxidases and to retain only peroxidases A6, A7 and A8. However, fractions 116 to 125 (containing A8) were pooled and kept without any further chromatography step.

Figure 5A shows a high peak surrounded by minor peaks, all obtained by elution with tris/HCl 75 mM. Figure 5B shows a first high peak followed by two minor peaks. The first high peak was obtained by elution with tris/HCl 105 mM and the two minor peaks by tris/HCl 120 mM. Fractions 35 to 39 (containing A6) of chromatography 5A and 27 to 34 (containing A7) of chromatography 5B were pooled separately.

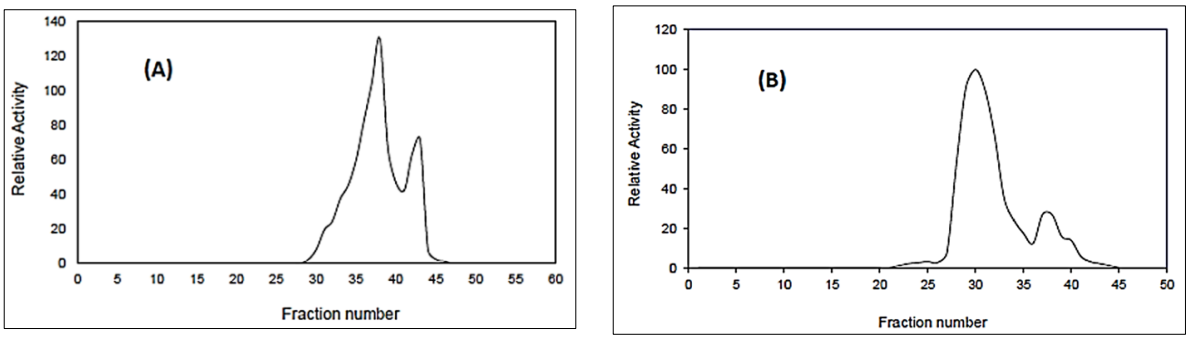

Figure 5 Elution profile of some shoots anionic isoperoxidases after ion exchange chromatography on DEAE-cellulose. (A): group of fractions containing A6 after the first chromatography; (B): group of fractions containing A7 after the first chromatography. Stepwise elution was carried out by increasing concentrations of tris/HCl buffer pH 8 (50mM, 75 mM, 90 mM, 105 mM, 120 mM, 135 mM and 150 mM)

The fractions pooled after the first and the two last chromatography were subjected to native electrophoresis, in order to demonstrate that isoperoxidases A6, A7 and A8 had been isolated from each other (Figure 6).

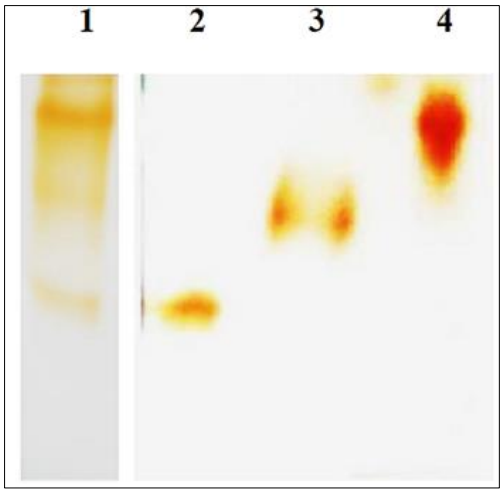

Figure 6 Native-PAGE of the shoots anionic isoperoxidases A6, A7 and A8 after ion exchange chromatography. Lane 1: anodic peroxidases of shoots crude extract; lane 2: isoperoxidase A8; lane 3: isoperoxidase A7; lane 4: isoperoxidase A6

4. Discussion

The activity of soluble peroxidases of the extracts of *Vigna* sp. seedling is easily detectable at the first days of germination. This activity intensifies as germination evolves, which suggests a role of these peroxidases in the plant growth. The increase in peroxidase activity per fresh matter weight is especially obvious before the 6th day of germination. Beyond this time of germination, the increase in their rate of expression seems less obvious, which could be explained by the fact that probably, after approximately 6 days of germination, the optimal peroxidase concentration necessary to the development of the seedling is reached.

It is proven that certain peroxidases are implied in plants morphogenesis [4, 15, 16, 17]. However after six days of germination, all the organs of the vegetative system were already appeared. The rate of peroxidases expression is then stabilized after this time of germination probably for this reason. An isoperoxidase (A6) in particular seems to be very implicated in this process of growth. Its activity is relatively great in all part of the whole seedling, compared to the other soluble isoperoxidases of *Vigna* sp. seedlings.

This great activity can be due either to a great catalytic efficiency with respect to the substrates used in this study (ABTS, O-dianisidine and H_2O_2), or to an important rate of expression of its gene. This isoperoxidase has moreover a very great

thermal stability. After incubation of crude peroxidase extracts of the seedlings of *Vigna* sp. for 10 min at 80°C and 85°C, the zymogram shows that a considerable amount of initial activity of this isoperoxidase is preserved. Such a heat resistance is rare among peroxidases of other plants, studies having shown that many peroxidases are completely inactivated at temperatures quite lower than 80°C [9, 11, 18, 19, 20]. Plotting Log (residual activity) vs. heating time after exposure to 80°C leads to a biphasic linear relationship for roots peroxidases. This observation confirms a differential inactivation kinetics of the numerous peroxidase isoenzymes present in that tissue. In shoots, the above-mentioned plot is monophasic, probably because there are less isoenzymes in this part of the seedling, and the heat inactivation kinetics is mainly due to the more expressed isoperoxidases, especially A6.

Some intermediate migrating anodic isoperoxidases seem to be as thermostable as A6, but either they are expressed in lower quantities, or have less catalytic efficiencies compared to the latter. A6 presents moreover compared to other thermostable isoenzymes the advantage of being highly expressed as well in roots as in shoots. It is then possible to obtain it in great quantity by extracting it from the whole seedling.

The great thermal stability and important activity of A6 in the seedlings of *Vigna* sp. make it possible to use it in many applications. For example, it may be used in ELISA techniques, where there are critical factors that limits the enzyme detectability, so that the specific activity of the enzyme used must be high, or the stability of enzyme-antibody conjugate should be optimal to allow longer storage durations [21].

One might be feared that the large number of isoperoxidases present in *Vigna* sp. extracts might complicate the separation of peroxidase A6 from other isoenzymes. However, this study has shown that starting from shoot extracts only, fewer isoenzymes are present, and peroxidase A6 can thus be isolated quite easily from the others by anion exchange chromatography. It is now necessary to carry out a more complete purification, separating it from any other contaminating protein, in order to be able to experimentally determine its physicochemical and catalytic characteristics.

5. Conclusion and perspectives

There are at least ten peroxidase isoenzymes in extracts of *Vigna* sp. seedlings. Some heat-resistant isoperoxidases were identified and could be separated by anion exchange chromatography on DEAE-cellulose. One of these heat-resistant peroxidases, named A6 in this study, shows in addition a strong enzymatic activity in these extracts. More complete purification and characterization of this potentially useful peroxidase in biotechnology can thus be envisaged in the near future.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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