Peroxidase isoenzymes from seedlings of *VIGNA sp* (V) landrace Vn: distribution and thermal stability

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Abstract

Several soluble peroxidase isoenzymes are expressed in a landrace of *Vigna sp c*ultivated 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 radicles than in shoots. They have different thermal stability, so that heat inactivation kinetics of crude peroxidase extracts from radicles does not fit the first-order model. One major anodic isoperoxidase of the slow migrating group and at least two others anodic isoperoxidases of the The slow and intermediate migrating groups of anodic isoperoxidasesanodic isoperoxidases are stable for ten minutes of incubation at 80°C and 85°C. The major anodic isoperoxidase of the The less anodic slow migrating groupisoperoxidase (named A6 in this study) shows in addition to this great thermal stability, a high activity during germination and is expressed both in radicles and shoots in large amounts. The combination of those characteristics makes thisthat isoperoxidase a a potential candidate for biotechnological applications.

Introduction

Peroxidases are present in nearly the totality of the living organisms, and are often found in the form of several isoenzymes, as the result of gene duplication mutations which occurred during evolution, and somewhat due to differential post-translational modifications. In plants, these isoperoxidases are expressed in different degrees depending on the stage of growth or nature of the tissue (Shannon, 1948; Stahord and Bravinder-Bree, 1972 Birecka and Miller, 1974;). These have various physiological roles in plant cells: they participate in different reactions including lignification, cross-linking of cell wall polysaccharides, regulation of cell elongation, wound healing, phenol oxidation or oxidation of indol-3-acetic acid (Gaspar et al., 1991). A lot of data pointed to the responsiveness of peroxidases to various biotic and abiotic stresses (Moerschbacher, 1992; Castillo, 1992; Esnault et Chibbar, 1997; Novakovic et al., 2018). While all these peroxidase isoenzymes have the 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 even among the isoenzymes in the same plant (Agostini et al., 1999; Rodriguez et al., 2002; Tipawan and Barret, 2005; Fernández-Fueyo et al., 2014).

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. The results revealed that the residual activities in this landrace were 67%, 34% and 3.4% at 70°C, 75°C and 80°C respectively after preincubation for 1 hour. This same landrace retained more than 55% of its peroxidase activity after preincubation for 3 weeks at 55°C and 47% after storage for 1 year at room temperature (Mbassi et al., 2011).

The present work aims at quantifying and stud tissue distribution and heat stability of isoperoxidases expressed during the germination of the above mentioned Vigna *sp* landrace.

Material And Methods

Reagents:

O-dianisidine (3,3'-dimethoxy benzidine), PMSF, EDTA, EGTA, 30% Hydrogen peroxide, sodium phosphate monobasic and dibasic, acrylamide and N,N'-methylene bis acrylamide, HEPES, imidazole, ammonium persulfate, TEMED, glycerol, bromophenol blue and methyl green were from SIGMA or SIGMA Aldrich. Concentrated HCl was from CARLO ERBA. Distilled water was used to prepare aqueous solutions.

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.

Germination procedure:

Seeds were germinated according to the same procedure previously described (Mbassi et al, 2011). Fifteen seeds were allowed to germinate in 4 Petri dishes. Samples of seedlings were collected after 3, 6 and 9 days respectively of germination. The fresh weight of each seedling was recorded. This biological material was stored at -20°C until use.

Protein extraction:

Four gram of each sample was treated as previously described to extract proteins (Mbassi et al., 2011). Crude extracts were used for subsequent electrophoresis and activity assays.

Activity assays:

The enzyme activity was measured by using 5µl of enzyme extract, 100µl of 0.5 mM hydrogen peroxide and 100µl of 1 mM Odianisidine in 100 mM acetate buffer pH 5 in a final volume of 2 ml. Activity was monitored by scanning the increase in absorbance of O-dianisidine oxidation product at 460 nm ($\varepsilon_{460\ nm}$= 11.3 mM^{-1} cm^{-1}) with a Biochrom Libra S12 spectrophotometer. Measurements were done at room temperature (~24-26°C).

Electrophoresis and gel staining:

Native or nondenaturing polyacrylamide gel electrophoresis was performed in 6% polyacrylamide gel with the continuous buffer system Imidazole/HEPES pH 7.4 described by McLellan (1982). Each well contained 2 µl of extract. Electrophoresis was carried out toward the anode to separate anodic isoperoxidases and toward the cathode to separate cathodic isoperoxidases. The gels were run at a constant voltage of 200 V.

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.

Isoperoxidases thermal stability assays:

Aliquots of 100 µl of peroxidase extracts from radicles 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 (0°C) for at least 5 min. Following centrifugation at 15 000 g for 10 min at 4°C, the supernatants were collected, and used as sources of peroxidases for gel electrophoresis. In another experiment, 100 µl of the supernatants were incubated in microfuge tubes for different times varying from 1 min to 10 min at 80°C. The residual activity of these samples was then measured. Log (A ⁄Ao) versus time plots were established (Ao is the initial enzyme activity and A is the residual activity after heating for time t).

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.

Results

Peroxidase activity during seed germination:

Table I shows the relationship of the weight of the starting fresh material and peroxidase activity of whole seedlings of *Vigna sp at different times of germination*. It is observed thatWed peroxidase activity increases relatively to the weight of the seedling during germination. This increase is more important between the 3rd and the 6th day of germination.

Table I: 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)	1.11±0.3 x 10^{-3} b,c	2.59±0.51 x 10^{-3} a	2.72±0.36 x 10^{-3} a

Expression and tissue distribution of peroxidases isoenzymes during germination:

We have evaluated by native electrophoresis the number of isoperoxidases that are expressed during germination of the Vn phenotype seeds. To determine the tissue localization of peroxidase isoenzymes, we separated, using a scalpel, the radicles and shoots of a 9-day-old sample of Vn landrace. Peroxidases were extracted from each part as described in the "Materials and Methods" section. The electrophoresis implemented on the one hand in the cathode-anode direction, and on the other hand in the anode-cathode direction revealed at least 10 major isoenzymes, depending on their relative mobility, whose expression rates are different (Fig. 1).

For anodic isoenzymes, there is two (named A6 and A5 in this study) slow migrating peroxidases (Rf = 0.1; 0,15) from which one (A6) is expressed in both radicles and shoots and A5 seems to be expressed only in radicles, two (A4 and A3) intermediary migrating isoenzymes (Rf = 0.25; 0.3) expressed in radicles, 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 radicles and one (A8) quick migrating isoenzyme (Rf = 0.38) expressed in shoots (Fig 1a). Concerning cathodic peroxidases, there are 2 isoperoxidases (C2 and C1) expressed in both radicles and shoots with low relative migrations. Moreover, their expression rate seems to be similar in both parts, given the similarity of 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 (Fig 1b).

Thermal stability of the different isoenzymes:

The results of heat stability experiments show that the slow and some intermediate migrating types of anodic isoperoxidases are stable when heat treated for 10 min at 80°C and 85°C respectively. After heating at 90°C for 10 min, the slow migrating isoenzyme still revealed some weak activity. vity. 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 (Fig. 2).

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

Discussion

The activity of soluble peroxidases of the extracts of *Vigna sp* seedling is easily detectable during the first days of germination. This activity intensifies as germination evolves, which suggests a role of these peroxidases in the growth of the plant. The increase in peroxidase activity per fresh matter weight is clearly obvious before the 6^{th} 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 has been attained

It has been demonstrated that certain peroxidases are involved in plants morphogenesis (Gaspar et al, 1991; Loukili et al, 1997; Cella and Carbonera, 1997; Petrić et al., 2015). However after six days of germination, the organs of the vegetative system were already all appeared. The rate of peroxidases expression is then stabilized after this time of germination probably for this reason. An isoperoxidase in particular seems to be very implicated in this process of growth (AA6). Its activity is detectable in the early days of germination, and in addition, this activity is relatively high throughout the germination process compared to the others soluble isoperoxidases of *Vigna sp* seedlings (data not shown).

This high activity can be due either to a great catalytic efficiency with respect to the substrates used in this study, or to an important rate of expression of its gene. This isoperoxidase has moreover a very high 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 heat-resistance is rare among the peroxidases of other plants; in fact many studies have observed that a lot of peroxidases are completely inactivated at temperatures quite lower than 80°C (Agostini et al, 1999; Khales and Baaziz, 2004; Tipawan and Barret, 2005; Navid et al, 2007, Sergio et al, 2007). A plot of the residual activity against the heating time after exposure to 80°C reveals non-linear relationship in radicles. This observation confirms differential inactivation kinetics of the numerous peroxidase isoenzymes present in that tissue. In shoots, the above-mentioned plot is linear, probably due to the fact that 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 isoperoxidases the advantage of being highly expressed as well in radicles as in shoots. It is then possible to obtain it in great quantity by extracting it from the whole seedling.

The high thermal stability as well as the important activity of A6 in the seedlings of *Vigna sp* make it's a potential tool in many applications. For example, it may be used in ELISA techniques, where the detection process requires enzymes of high specifity as well as an optimum of the stability of the enzyme-antibody conjugate so as to favour longer storage (Vierling et al, 2000).

Declarations

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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Figures

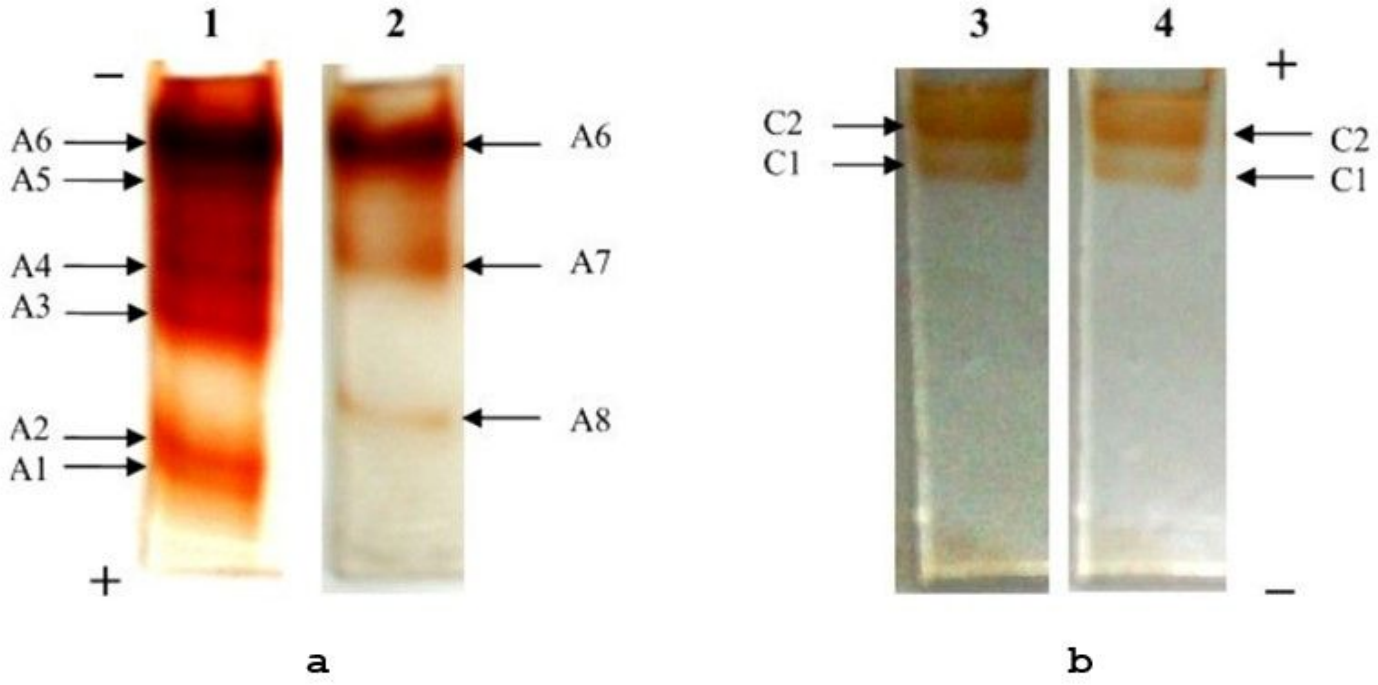

Figure 1

Distribution of peroxidase isoenzymes among radicles and shoots of seedlings from Vigna sp. Lane 1: anodic isoenzymes of radicles; Lane 2: anodic isoenzymes of shoots; Lane 3: cathodic isoenzymes of radiclesradicles; Lane 4: cathodic isoenzymes of shoots.

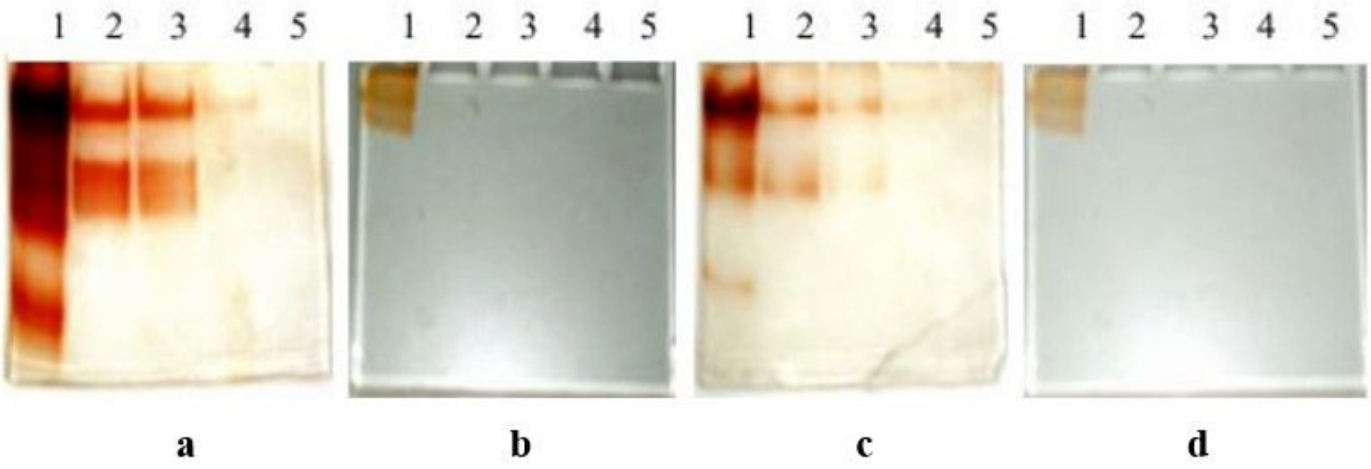

Figure 2

Thermal stability of soluble peroxidase isoenzymes from Vigna sp. expressed after 9 days of seedling growth. (a) anodic isoenzymes of radiclesradicles; (b) cathodic isoenzymes of radiclesradicles; (c) anodic isoenzymes of shootsshoots; (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.

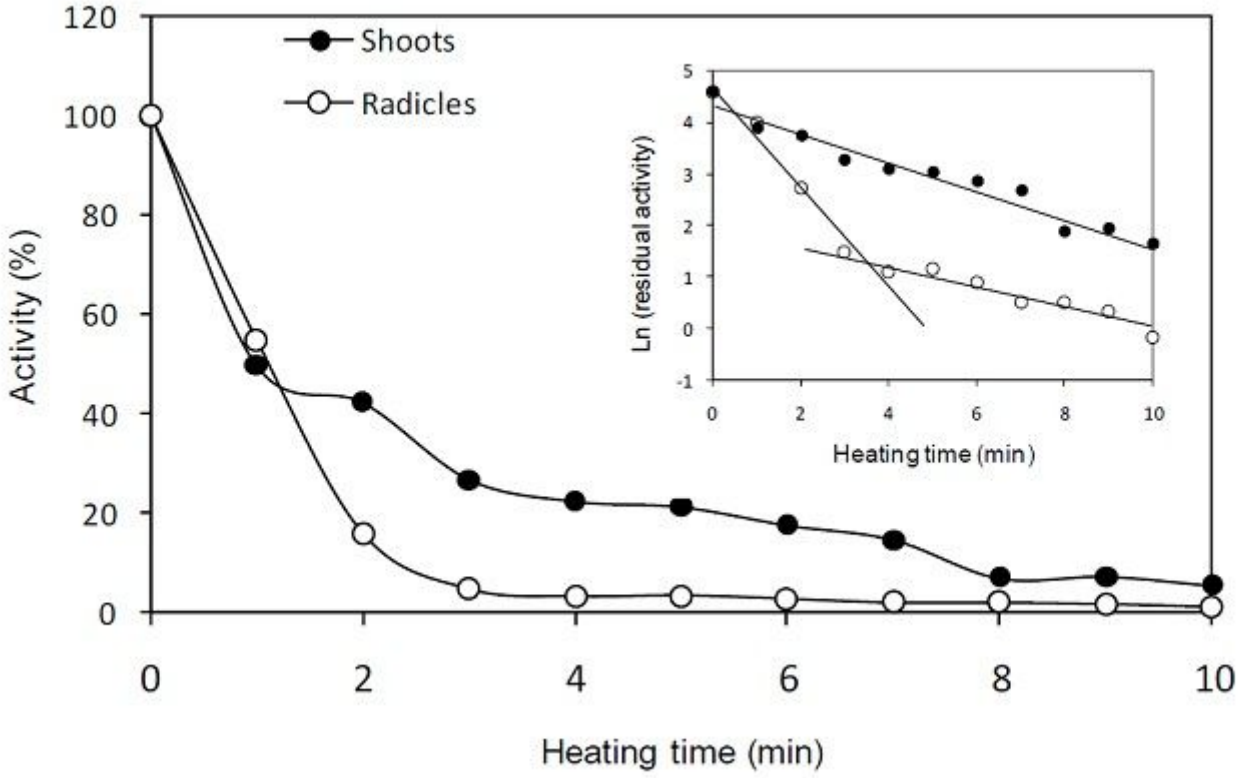

Figure 3

Kinetics of thermal inactivation (at 80°C) of crude extracts of soluble peroxidases of radicles 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 %.