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Original Research Article

Comparative Analysis of the Kinetic Properties of Peroxidases from two Improved Sorghum Varieties

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ABSTRACT

Varietal differences and their influence are not often critically considered when assessing properties of industrially important enzymes such as peroxidases, from cereals. Therefore, this study aims at closing that knowledge gap, by purifying peroxidases from two improved sorghum varieties, KSV8 and SK5912, which are food and brewery-grade grains used extensively all over the world. This was done via $(NH4)_2SO_4$ precipitation, ion exchange chromatography and gel filtration, and comparing their properties. Both KSV8 and SK5912 peroxidases had their soret peaks at 402 nm but with different extinction coefficients (ε_{402} = 113 mm^{-1} cm^{-1} for KSV8 and ε_{402} = 119 mm^{-1} cm^{-1} for SK5912; while their molecular weights using ESI mass spectral data were 35.591 and 35.645 kDa, respectively. Optimum activity pH for KSV8 were pH 5 (guaiacol and o-dianisidine) and pH 4 for ABTS, pyrogallol pH 8, while for SK5912, they were pH 5 (guaiacol) and pH 4 (o-dianisidine, ABTS and pyrogallol). Optimum temperatures when measured with guaiacol were 50^{0}C with KSV8 and 40^{0}C for SK5912. Steady state kinetics of the enzymes with the same organic compounds showed that they have different affinities for them with o-dianisidine being the most efficiently oxidized substrate. Calcium greatly increased the activities of both sorghum peroxidases; however, the rate was much higher in SK5912 than in KSV8, while with ferric ion, the opposite obtained. Such differences or similarities were replicated across several other properties studied. These findings underline the great importance of differences in cereal varieties which should be reflected in their different biotechnological applications.

Keywords: Cereal enzymes, Enzymes and catalysis, Food and brewing chemistry, Protein purification, Sorghum and cereals, Sorghum peroxidase.

Introduction

Cereals have been described as the 'staff of life' as they have since the dawn of civilization been the source of many of mankind's most important foods and other materials including enzymes.[1] Researchers have been interested in enzymes from cereals because of the important physiological roles they play in such plants which could be exploited for industrial purposes. Sorghum (*Sorghum bicolor* L. Moench) is an important C4 cereal used all over the world for many nutritional and industrial purposes, similar to the other major cereals, such as corn, rice, wheat and barley. However, sorghum has a major cultivation advantage over these other cereals due to the fact that it is very drought-resistant and able to grow and thrive well in semi-arid, drought-ridden and difficult terrains that are too harsh for other domesticated cereals and plants to survive in.[2,3] Believed to have originated in Africa about 5,000 years ago, sorghum spread from there after domestication to other parts of the world, first to India, then to China and afterwards to the Middle East, Europe and other parts of the Western hemisphere.[4]

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There are presently over 10,000 different cultivars or varieties of sorghum available, and efforts are on-going to increase their number and types through different plant breeding experiments.[5-7] Sorghum is a staple food in many parts of Africa, Asia, Central America and the Middle East and used as the main source of beverages in many of these places.[8] Among the wide range of food products made from sorghum are bread, pancakes, dumplings and couscous, porridge, gruels, cakes, cookies, pasta, a parboiled rice-like product, snack food and also beer.[4] Importantly, food products from sorghum are safe for consumers with celiac disease and are thus ideal for gluten-free preparations.[9,10]

Many important enzymes also occur in sorghum; among them are peroxidases.[11] Plant peroxidases are important widely occurring proteins in the plant kingdom that use H_2O_2 to oxidize a large variety of hydrogen donors such as phenolic substances, amines, ascorbic acid, indole and also inorganic ions.[12] Interest in peroxidases from sorghum arise from the fact that peroxidases as antioxidant enzymes play important roles in preventing peroxidation of many types of food products such as beer, porridges, couscous etc. made from sorghum, and in that way extend their shelf lives.[13] A few persons have previously purified peroxidases from sorghum.[11,14-16] Considering the enormity of sorghum varieties available, these few works are not enough to explain the many properties that could account for the impact associated with varietal differences on the expression of an important enzyme as peroxidase. In most of these works, not only are differently colored varieties typically used, they come from different climes and countries, a situation that could impact on the properties of the crop including those of important metabolic enzymes such as peroxidases that occur in them. Sorghum grains KSV8 and SK5912 are two varieties that have enjoyed tremendous usage and extensive

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applications within and outside Nigeria in such diverse areas as brewing, food and enzyme-related researches.[6] Previously, a cationic peroxidase from variety SK5912 was characterized.[11] However, that work was biased towards structural studies and so did not explain the properties of peroxidases from other sorghum variety in a general sense. Therefore, in the present study, peroxidase was purified from the white colored and widely used variety KSV8[5,6,17] and also from SK5912 in order to carry out a comparison of the properties of peroxidase from the two sorghum varieties. The aim was to help delineate the peculiar characteristics of peroxidases from different sorghum varieties as against the typical trend of just grouping them together. Such delineations apart from helping to define which varieties are best suited for different purposes and applications in the food and related industries, could have implications that may extend to other plant enzymes.

Materials and Methods

Grain preparation

The sorghum grain varieties KSV8 and SK5912 used for this work, were originally bought from Ahmadu Bello University, Zaria Nigeria (Institute for Agricultural Research, IAR) in August, 2010. The sorghum grains were duly identified and authenticated by Mr. Chijoke Onyeukwu, the curator of the Herbarium of the Department of Plant Sciences and Biotechnology (PSB) of the University of Nigeria, Nsukka. The voucher number of sorghum plant in the Herbarium is UNH 851. After being manually cleaned and sorted, the grains were sterilized as described by Ogbonna *et al*.[17] using a solution of hypochlorite (1%, v/v) and evenly spread on a surface previously cleaned and layered with soft absorbent papers. The setup was then allowed to dry at room temperature overnight.

Purification of sorghum peroxidase

Peroxidase was isolated and purified as described in an earlier study.[11] Apart from crushing the sorghum grains to a fine powder with a blender, all the purification steps were carried out at 4^{o}C. Proteins were extracted by incubating the crushed powder (200 g/L) with sodium phosphate buffer (100 mM, pH 6.0) for 30 minutes. These were then centrifuged at 5000 rpm for 30 minutes in an SLC 6000 Sorvall Evolution centrifuge. The crude proteins obtained were used for further work which first involved precipitation on ice, with ammonium sulphate up to 90% saturation with centrifugation, re-dissolution of the precipitates in phosphate buffer (50 mM, pH 8.5) and dialyzing in the same buffer overnight. After dialysis the dialyzed protein was applied to an anion exchange DE-52 DEAE cellulose column (2.5 x 15 cm) equilibrated with phosphate buffer (50 mM, pH 8.5) and washed with the equilibration buffer at a flow rate of 150 mL/h. The peroxidase did not bind to the anion exchanger but the resin retained a lot of non-peroxidase proteins (no protein activity shown, see below) but was eluted in few 10 mL fractions, pooled together and then concentrated by centrifuging at 4000 rpm with a 10 kDa MWCO membrane tubes in a centrifuge (SLC 6000 Sorvall Evolution) for 15 minutes. The concentrated protein was then dialyzed against 50 mM phosphate buffer (pH 6.0) before loading onto a 2.5 x15 cm CM-52 CM cellulose cation exchange column that have been equilibrated with the equilibration buffer (50 mM phosphate buffer, pH 6.0). The equilibration buffer was also used to wash the column at the flow rate 90 mL/h. Bound proteins were then eluted using a linear gradient of 0 – 0.6 M NaCl (200 mL, in equilibration buffer) at the same flow rate. Thereafter small fractions (5.0 mL) of the eluates were collected and monitored for peroxidase activity. A further chromatographic step (gel filtration) was carried out and used for analytical works that required higher levels of enzyme purification such as reduction potential (see below).

Protein and heme quantification and peroxidase activity determination

During the purification process, the presence of proteins was assessed by monitoring the absorbance of the fractions at 280 nm. The Bradford method using bovine serum albumin (BSA) as standard was used to quantify the proteins.[18] The pyridine hemochromogen assay was used to determine heme concentration.[19] Heme was also evaluated during the purification process by monitoring the absorbance of the fractions at 402 nm. Peroxidase activity was assessed by measuring the absorbance change at 470nm of guaiacol (Sigma chemicals co.) as it is oxidized to tetraguaiacol by the enzyme. The final reaction mixture contained 0.06 µM enzyme, 1.5 mM guaiacol, 0.5 mM H_2O_2 and 100 mM sodium phosphate buffer, pH 6.0. The assay was performed at 25^{o}C using a UV-Vis scanning spectrophotometer (Perkin Elmer Lambda 35) with temperature control. Absorbance increase at 470 nm was monitored for 90 seconds and the slope of the initial linear part of the curve used to determine activity. Enzyme activity was calculated using an extinction coefficient of 22.6 mM^{-1} cm^{-1} for tetraguaiacol.[20]

SDS-PAGE, and mass spectroscopy

The purity and molecular weight of the enzyme was assessed using SDS-PAGE under denaturing conditions.[21] Mass spectrometry (ESI) was used to more accurately determine the molecular weight(s) and confirm the integrity of the protein sample by excising the bands of interest from SDS-PAGE.[22]

Effects of temperature and pH on peroxidase activities

The effects of temperature and pH on the activities of the purified sorghum peroxidases were determined using a modification of the methods described by Suzuki *et al*.[23] The temperature activity profile was determined over a range of 30 – 90^{o}C by incubating a mixture of the enzyme and buffer for a period of 30 minutes in a water bath (Grant, England), before the substrates guaiacol and H_2O_2 were added to initiate reaction as described above. The effect of pH on the activity of the purified enzyme was studied over a pH range of 2 to 12.0 using the following buffers: 0.1M glycine-HCl buffer for pH 2; 0.1M phosphate-citrate buffer for pH 3 to 5; 0.1M Na-phosphate buffer for pH 6 to 8; 0.1M glycine-NaOH for pH 9 and 10 and 100mM Na-phosphate buffer for pH 11 and 12. The enzyme and appropriate buffer for each pH was mixed and equilibrated for 60 seconds before the substrates were added and absorbance change monitored as outlined above.

Steady state kinetic assays

Steady state kinetic studies of the enzymes were performed using different organic substrates and measuring the initial velocity rates of their activities in sodium phosphate buffer (100 mM, at the optimum pH obtained for each of them above which were between 4 and 8) at 25^{o}C. The substrates, which included guaiacol, o-dianisidine, ABTS and pyrogallol, were freshly prepared and their enzyme activities assayed using standard methods.[22,24] Initial rates of oxidation for each substrate was measured at the appropriate wavelength (guaiacol, ε_{470} = 22.6 mM^{-1} cm^{-1}; o-dianisidine, ε_{460} =11.3 mM^{-1} cm^{-1}; ABTS, ε_{405} = 36.8 mM^{-1} cm^{-1}; pyrogallol, ε_{420} = 12 mM^{-1} cm^{-1}). All reactions were initiated by the addition of 0.5mM concentrations of H_2O_2. Values for K_m and k_{cat} parameters were determined by fitting nonlinear least-squares of the experimental measurements, from Lineweaver-Burk plots to the Michaelis-Menten equation using Microsoft Excel.

Ligand binding studies

To determine the equilibrium binding parameters of the sorghum peroxidases, different microlitre amounts of suitably diluted ligand (cyanide) solutions were added to filter sterilized 100 mM sodium phosphate buffer, pH 7.0 in a cuvette containing the enzymes. Thereafter, the cuvette and contents were mixed by inversing it several times and allowed to equilibrate. The UV-visible spectra (270 – 700 nm) were recorded for the various dilutions of the ligand. The binding affinity of cyanide was monitored spectroscopically at 419 nm.

Reduction potential determination

The ferric/ferrous reduction potential of the enzyme was determined as previously reported[11,25] using the xanthine/xanthine oxidase method. Potassium phosphate buffer (50 mM, pH 7.0) was made oxygen free by using glucose (5 mM), glucose oxidase (50 µg/ml) and catalase (5 µg/ml). Thereafter, phenosafranin, which is a dye of known potential, (E^{o}, = -252 mV) was used.[26]The assay mixture also included xanthine (300 µM), xanthine oxidase (50 nM), benzyl viologen (0.2 µM), enzyme (20 µM). Absorbance changes which correspond to the reduction of heme were then measured at the isosbestic point of phenosafranin (407 nm). Reduction of the dye was measured at 520

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nm, at which point the change caused by heme reduction had become negligible. Linear Nernst plots for one-electron reduction of heme (25 mV In(D_{ox}/D_{red}), and the two-reduction of dye (12.5 mV In(D_{ox}/D_{red}), where E_{ox}, E_{red} and D_{ox}, D_{red} are the concentrations of oxidized and reduced forms of enzyme and dye, respectively, produced the expected slope of 1 over a wide range of potentials, with the intercept giving a reliable value for $\Delta E^{o\prime}$ with an error margin of ± 2 mV.

Effect of metal ions on peroxidase activities

The effect of various metal ions (Ca^{2+}, Fe^{3+}, k^{+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Rb^{+} and NH_4^{+}), chelating agents (EDTA and DTPA) and inhibitor (ferricyanide) on peroxidase activity was determined by pre-incubating 10 mM concentrations of each of them with the enzyme and buffer for 10 minutes before standard peroxidase assay.[23]

Statistical analysis

The values for K_m and k_{cat} parameters were determined by fitting nonlinear least-squares of the experimental measurements, from Lineweaver-Burk plots to the Michaelis-Menten equation using Microsoft Excel. All the analyses were carried out in triplicates.

Results and Discussion

This work reports the purification and characterization of cationic sorghum peroxidase from two Nigerian sorghum varieties, KSV8 and SK5912, both of which are widely used for food related research.[4-6,17] Previously peroxidase was purified from variety SK5912.[11] However, several important properties such as effects of pH, influence of metals and inhibitors were not part of that study. Instead, it had dwelt more on the structural and related spectroscopic aspects of the enzyme, whereas the present study, is an investigation of some biochemical parameters. Additionally, the present work attempts to compare these parameters in peroxidase from two sorghum varieties. The different properties (grain and malt quality) of the two improved sorghum grain varieties have also been discussed.[6] Purified peroxidase from sorghum varieties KSV8 and SK5912 were obtained using a 0-6M NaCl salt gradient from the CM-52 cation exchange resin. Thus, the obtained peroxidases were cationic. The purified enzymes were obtained as a single peak between the 4th and 12th fractions and 4th and 10th fractions for the two varieties respectively. Table 1a and 1b show that four purification steps were used to purify and subsequently obtain homogenous enzymes from the two sorghum varieties. At the end of purification, a purification fold of 29, a yield of 26%and a specific activity of 262 U.mg^{-1}was obtained for KSV8 while for SK5912 values obtained were 28-fold purification, 30% yield and specific activity of 282 U.mg^{-1}. The tables show that the enzymes were purified to homogeneity using just ammonium sulphate precipitation and ion exchange chromatography. This is a relatively minimal number of purification steps which is desirable during enzyme purification, as it reduces the immense cost often associated with protein purification.[27] The ease of purification was ensured by the 3-step precipitation using ammonium sulphate, a process which ensured the elimination of other proteins and left only those able to bind to the cation resin (CM-52 carboxymethyl cellulose) used in the work. As a matter of fact, the anion resin used probably retained other non-peroxidase proteins as the final cationic peroxidases did not bind to it. Despite its success some analytical work needed much higher level of purity than the above (such as reduction potential assay and EPR spectroscopy (not shown)) and thus a third level of chromatographic purification (gel filtration) was introduced to accommodate them. From the SDS PAGE gel results, protein bands were taken and their molecular weights accurately determined and confirmed via electron spray ionization mass spectroscopy. A monoisotopic value of 35.570 kDa was obtained for KSV8 while for SK5912 the value obtained was 35.647 kDa (Figure 1). The results also show that the enzymes essentially exist in multiple isoforms all falling within the molecular mass range of 35 kDa. These molecular weight measurements show in essence that the peroxidases obtained from the two sorghum varieties are similar to one another and therefore could be isoforms. The results are also similar, although with slight differences, to those obtained by Dicko *et al*. (2006) via MALDI-TOF mass spectrometry (34.283 – 35.631 kDa); but differed more widely from those reported previously by others.[14,15]Sae *et al*.[14] had reported a molecular mass of 43 kDa while Omdiji *et al*.[15] reported a molecular weight of 63 kDa, although they worked with malted sorghum and not the unmalted type used in this work. ESI mass spectroscopy data also shows that the sorghum peroxidases may exist in multiple isoforms all of course falling within the molecular mass range of 35 kDa. Typically, ESI mass spectroscopy increases or decreases by one proton over the course of its distribution across a spectrum. However, the existence of different bands with their different individual peaks could well suggest that they are isoforms of the proteins. Of course, the existence of different isoforms of peroxidases is now common knowledge.[28,29] Another important pointer to the possibility of isoforms among sorghum peroxidases is the fact that different optimum pH and temperature were observed for the peroxidases we report here and some others we had also purified,[30] although they were similar in many other details such as molecular weight and EPR spectra among other properties.

The UV-visible spectrum of peroxidase from the two sorghum varieties is shown in Figure 2. Both enzymes had their soret peak at 402 nm and additional absorption peaks at 496 and 636 nm, but differed in their molar absorptivity values (ε = 113 mM^{-1} cm^{-1} for KSV8 and ε = 119 mM^{-1} cm^{-1} for SK5912). The UV-visible electronic spectra of the sorghum peroxidases presented represent the typical peroxidase spectra that have been published previously.[11,16,31] The spectra show a soret maximum that falls within the range usually associated with peroxidases and similar enzymes which usually lies in the region of 400 nm. However, unlike what was reported previously for sorghum peroxidase,[16] the peroxidases studied here absorbed maximally at a wavelength of 402 nm, instead of 403 nm, while its β-band showed at 496 nm, instead of 497 nm as was reported. They are also somewhat different from the 498 nm we reported previously (Nnamchi *et al*., 2016). Slight variations in soret maxima have been reported to occur between otherwise similar peroxidases.[32] Higher or lower wavelength is indicative of the nature of the spin and coordination state of the enzyme. Such variations, however, become significant when they exceed 2-3 nm and so the small variations observed here are not significant enough to be considered as differences as such. The UV spectrum of the sorghum peroxidase shown here is clearly indicative of a high spin heme protein. Our sorghum peroxidase soret maxima also differ from those that have been reported for different plant peroxidases, such as that reported for barley peroxidase which was 399 nm.[33] In Figure 3 we present the effect of different hydrogen ion concentrations (pH) and temperature on the activities of KSV8 and SK5912 sorghum peroxidases with different organic substrates. The use of different substrates appeared to affect the optimum activity pH values given by the enzymes. For variety KSV8, the optimum pH with guaiacol was pH 5.0 (Figure 3a), while with o-dianisidine (Figure 3b) the same pH value of pH 5.0 was also obtained. With pyrogallol (Figure 3c) the optimum pH was 8.0, while with ABTS (Figure 3d), the optimum activity pH was 4.0. In the case of variety SK5912, optimum pH obtained with guaiacol was also 5.0 (Figure 3e), while with the other substrates o-dianisidine, pyrogallol and ABTS the same value of 4 was obtained for all (Figure 3f-h).

Several somewhat similar or different pH optimal values as obtained here have been reported by different authors. Nwanguma and Eze[13] had found that the optimum activity of some sorghum peroxidase with o-dianisidine had occurred at pH 5.5 which is close to that found in the present work. Bestwick *et al*.[34] similarly reported different optimum activity pHs with different substrates used to assay lettuce leaf peroxidases. They found that the pH optima for this peroxidase were respectively 4.5, 6.0, 5.5-6.0 and 6.0-6.5 for the substrates tetramethylbenzidine, guaiacol, caffeic acid and chlorogenic acid. Similar results were also reported by Nielsen *et al*.[35] with highly similar peroxidases obtained from the plants *Arabidopsis thaliana* and horseradish peroxidases A2. Suzuki *et al*.[23] working with buckwheat peroxidase, reported optimal pH values for peroxidase isoforms I and II as 9.0 and 9.0 (with guaiacol), 6.0 and 5.0 (with o-dianisidine) and not detected and 3.0 (with ABTS).

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Table 1a: Purification table of KSV8 sorghum peroxidase

Purification step	Total activity (U)	Total Protein (Mg)	Specific activity (Umg^{-1})	Yield (%)	Purification fold
Purification fold	24121	2598	9.	100	1
60-90% ASP	16205	845	19	67	2.1
DEAE-52 Anion exchange chromatography	9937	441	23	41	2.6
CM-52 cation exchange chromatography	6281	24	262	26	29

Table 1b: Purification table of KSV8 sorghum peroxidase

Purification step	Total activity (U)	Total Protein (Mg)	Specific activity (Umg^{-1})	Yield (%)	Purification fold
Purification fold	21420	2105	10	100	1
60-90% ASP	15121	890	17	71	1.7
DEAE-52 Anion exchange chromatography	9467	430	22	44	2.2
CM-52 cation exchange chromatography	6498	23	282	30	28

More relevant to the current study however, is the report of Dicko *et al*.[16] who had purified a cationic peroxidase similar to ours from sorghum. They had found that the enzyme had optimum pH for the substrates ABTS, ferullic acid and N-acetyl-1-tyrosine at pH 3.8, 5.5 and 6.5, respectively. Our results are close to these with ABTS, for example, consistently giving a pH optimum of 4.0. It is thought that these differences in activity of different pHs with different substrates may reflect the dependence on pH of their different ionization potentials.[16]

On how temperature affect the activities of the enzymes, the optimum value obtained for KSV8 sorghum peroxidase was 50^{o}C (Figure 4a), while the value obtained for SK5912 was 40^{o}C (Figure 4b). These temperatures are obviously high and classify them as stable and therefore, industrially desirable. As a matter of fact, the stability associated with sorghum and other plant peroxidases is defined even more in terms of heat and thermotolerance. Nwanguma and Eze[13] emphasized that property and also, their ability to regenerate after being heat inactivated. This is a useful property as it means that they will be less amenable to denaturation. Of course, it is a known fact that one industrial disadvantage of a lot of commercially useful biocatalysts is their relative low stability.[36]

The steady state kinetics of the two peroxidases from KSV8 and SK5912 were performed with the substrates o-dianisidine, pyrogallol, ABTS and guaiacol. Results obtained showed that in all cases the two cationic peroxidases obeyed Michaelis-Menten kinetics and are presented in Table 2. In both cases, o-dianisidine proved to be the most efficiently oxidized of the substrates with the Kcat value of 156 s^{-1} and very high K_{cat}/K_m value of 745 given by KSV8, while SK5912 gave lower values of Kcat 89.8 ± 0.9 s^{-1} and K_{cat}/K_m of 449 for o-dianisidine. The same Km value of 0.2 was also obtained with o-dianisidine with both peroxidases from the different sorghum varieties. However, the lowest K_m value obtained among all the assayed substrates of the two sorghum peroxidases was reported for KSV8 pyrogallol (0.11 mM); while the highest K_m value was obtained for KSV8 guaiacol (2.10 mM). In terms of overall best performance, O-dianisidine was the best substrate for both sorghum peroxidases, followed by pyrogallol, ABTS and lastly, by guaiacol. Thus, o-dianisidine showed the highest catalytic efficiency (Kcat/Km) amongst all the substrates, even though guaiacol was used as the main substrate and indicator for peroxidase activity. This was because guaiacol is a universal substrate for peroxidases; it is typically oxidized by all known peroxidases, hence the oft-mentioned reference to some peroxidases as guaiacol-type peroxidases.[23,37] It should also be noted that the catalytic efficiency data presented here were calculated using the extinction coefficient value of 113 mM^{-1} cm^{-1} for KSV8 and 119 mM^{-1} cm^{-1} for SK5912 which we obtained using the pyridine hemochromogen method of Antonini and Brunori.[11]

Presented in Figure 5 are the results obtained during ligand binding studies with potassium cyanide. Cyanide bound very well to the two sorghum peroxidases. At the assay wavelength of 419 nm the corresponding extinction coefficients obtained gave the binding constant values of 11.9 µM and 10.9 µM for SK5912 and KSV8 sorghum peroxidases respectively. Typically, ligand binding to heme peroxidases gives information that show the molecular processes used by the protein environment to control the interactions of its heme components with exogenous molecules, giving such information as heme accessibility, electronic structure, general heme protein conformation and reversibility of molecular binding to heme proteins among others.[22,31] The intimate binding of the two peroxidases are expected because most plant peroxidases typically bind to cyanide.

Result obtained for reduction potential of the two peroxidases from sorghum is shown in Figure 6. The figure shows that they both have similar graph patterns. Both KSV8 and SK5912 sorghum peroxidases also gave the same low reduction potential values of -266 mV for the Fe3+/Fe2+ reduction potential measurement. This is an obvious indication that peroxidases obtained from different sorghum varieties share a lot of properties commonly, but equally show differences in many other key properties such as optimum pH, optimum temperature, extinction coefficient among many others. Early reference was made to the importance of reduction potential in brewery control by Siebel and Singruen in 1935.[38] Recent references had centered on the contributions of polyphenols to reduction potential in beer and brewing, especially in relation to the containment of colloidal and flavor instability in beer and related beverages.[39] Our data for the Fe(III)/Fe(II) couple of KSV8 and SK5912 sorghum peroxidases fall within the ranges which had been reported previously for different plant peroxidases (all against standard hydrogen electrode, SHE): turnip peroxidase, -222 mV; HRC-C, -254 mV; cucumber basic peroxidase, -320 mV and soybean APX native, -159 mV.[31,32]

Table 3 shows how some metal ions and chemical inhibitors affect the activities of KSV8 (Table 3a) and SK5912 (Table 3b) sorghum peroxidase. The first observation is that only a few of the metallic ions increased the rate of reaction of sorghum peroxidases. The metallic ion that induced the most change was Ca^{2+}, as it increased the activity of KSV8 sorghum peroxidase by over 1100% (eleven times) and that of SK5912 by a whopping 3500% (thirty-five times)! For KSV8, the next cation after Ca^{2+} to induce high peroxidase activity was Fe^{3+}; Fe^{3+} caused an activity level that was at par (slightly higher actually) with that of Ca^{2+} (over 1100% or 11.6 times). Even the supposed inhibitor DTPA slightly caused an increase in activity. The following metallic ions Cu^{2+}, Co^{2+}, K^{+}, Rb^{+} and Na^{+} caused some decrease in activity. With respect to SK5912 sorghum peroxidase, Cu^{2+} effected the next highest increase in enzyme activity, as it caused increased activity by about 675% (6.75 times).

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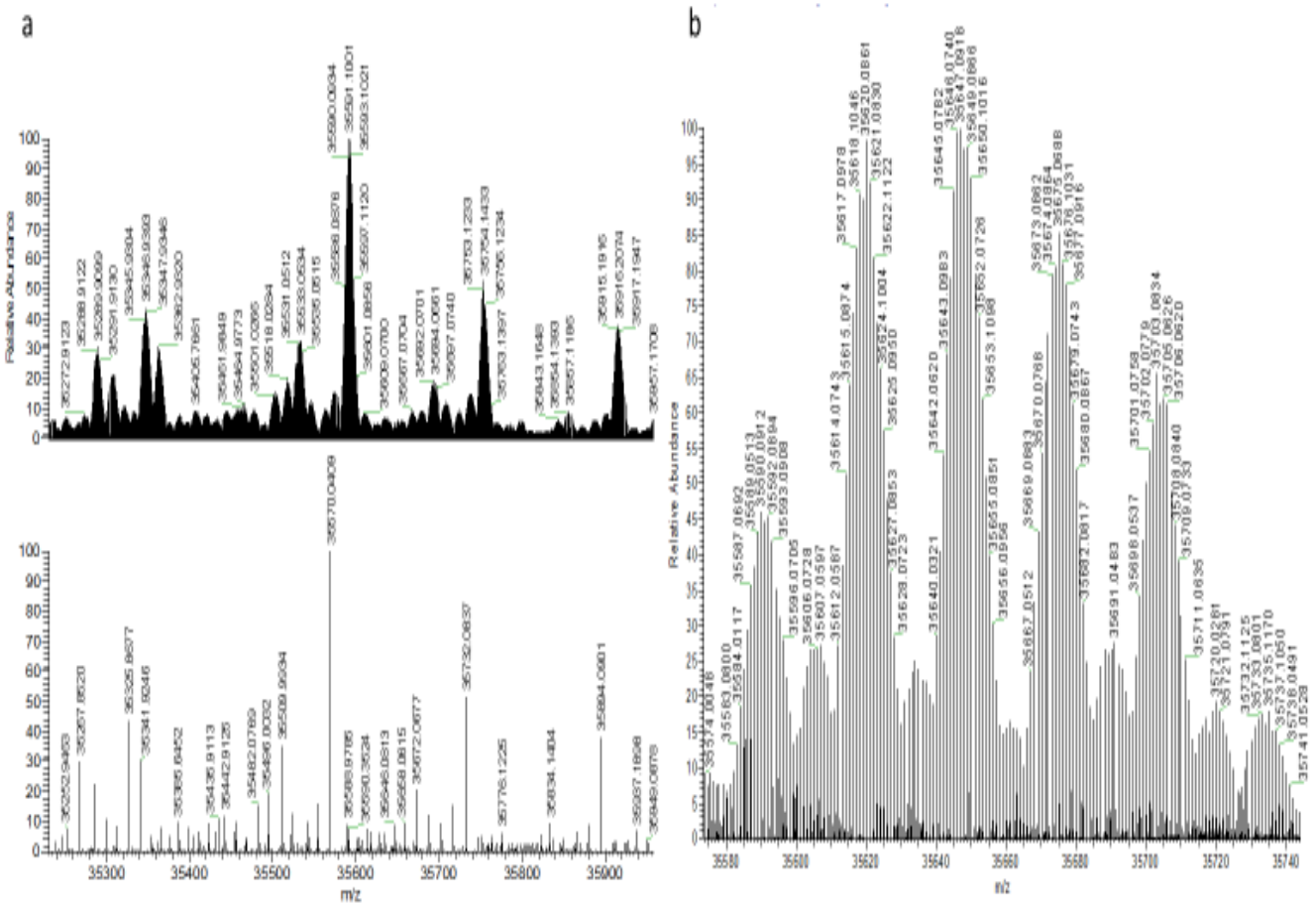

Figure 1: Orbitrap FT High Resolution ESI mass spectroscopy of KSV8 (a) and SK5912 (b) sorghum peroxidase showing their molecular weights

It was followed by Fe^{3+} while monovalent ions like Na^{+} and Rb^{+} lowered the activity of SK5912 sorghum peroxidase. EDTA, a metal chelator which often acts as an inhibitor of metalloenzymes lowered activity here slightly, but not so DTPA (diethylene-triamine-penta-acetic acid), another chelating agent which actually increased activity slightly. The importance of metals in protein and enzyme systems derives from the fact that approximately half of all proteins contain a metal.[40] Furthermore, it has been estimated that about a quarter, to a third of all proteins require metals to carry out their functions.[41]Scrimgeour[42] put it more directly by stating that roughly more than one-quarter of known enzymes require metallic cations to achieve full catalytic activities. Such functions in the main include enzymatic catalysis as well as transport, storage and signal transduction duties. With respect to catalysis, ionic interactions between enzyme-bound metals and substrates help put the substrates in the right orientation to enable reactions occur, or effect the stability of charged reaction transition states. Furthermore, metals can affect oxidation-reduction reactions by causing reversible changes in the oxidation state of metal ions. With this host of possible functions, it is not surprising to observe that different metallic ions had varying effects on the activities of the two sorghum peroxidases studied here. Furthermore, the higher activities due to the presence of calcium, which were as high as 3500% and 1100% respectively in SK5912 and KSV8 sorghum peroxidases point to the possibility that calcium play significant roles in sorghum peroxidases. According to Penel, [43] peroxidases are dependent on calcium ion through many mechanisms. Calcium mediates many different cellular processes, acting as secondary messenger and regulating many functions of plant cells.[44] The dependence of peroxidases on calcium is thought to be one reason why they are so reactive.

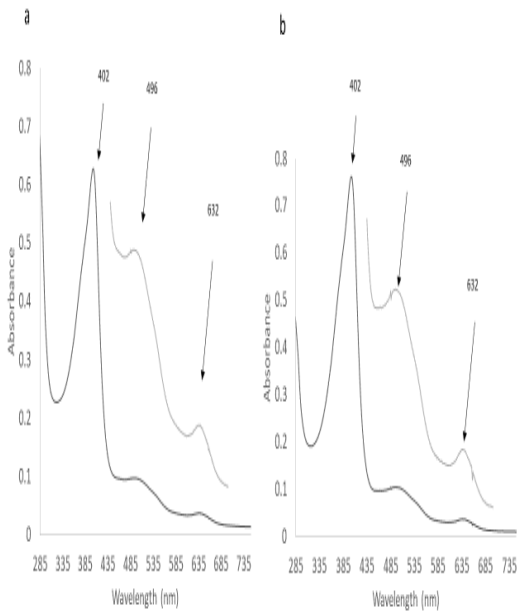

Figure 2: UV-visible spectra of KSV8 (c) and SK5912 (d) sorghum peroxidase (the absorbance scale in the visible region (435 – 685 nm) has been multiplied by five; Conditions: phosphate buffer, pH 6.0, μ = 0.05M)

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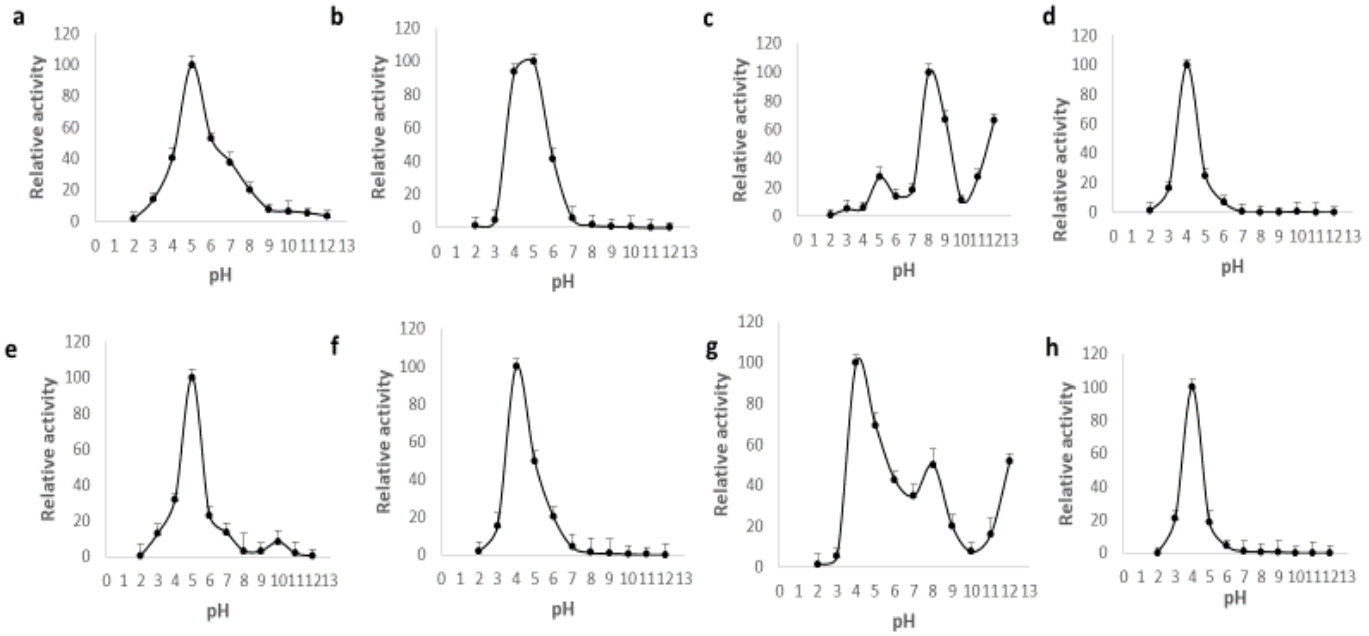

Figure 3: Effect of different pH on the activity of KSV8 sorghum peroxidase with guaiacol (a), o-dianisidine (b), pyrogallol (c) and ABTS (d) (optimum pH for each substrate is a = 5, b = 5, c = 8, d = 4) and SK5912 sorghum peroxidase with guaiacol (e), o-dianisidine (f), pyrogallol (g) and ABTS (h) (Optimum pH for each substrate is e = 5, f = 4, g = 4, h = 4)

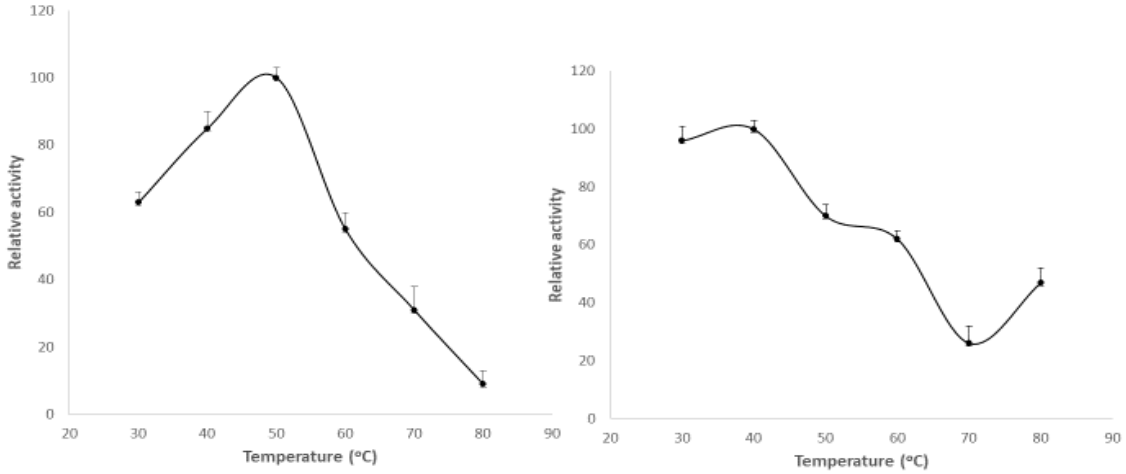

Figure 4: Effect of different temperature on the activity of KSV8 (a) and SK5912 (b) sorghum peroxidases; optimum temperature was 50^{o}C and 40^{o}C respectively for the two varieties

Table 2a: Reaction kinetics of SVS sorghum peroxidase organic substrates

Substrate (mM/s)	Kcat (S^{-1})	Km (mM)	Kcat/Km
Guaiacol	8.7	2.10	4.4
O-dianisidine	156.4	0.21	744.76
Pyrogallol	16	0.11	145.45
ABTS	16.10	2,03	7.90

Table 2b: Reaction kinetics of SK5912 sorghum peroxidase with different organic substrates

Substrate (mM/s)	Kcat (S^{-1})	Km (mM)	Kcat/Km
Guaiacol	8.7	2.10	4.4
O-dianisidine	156.4	0.21	744.76
Pyrogallol	16	0.11	145.45
ABTS	16.10	2,03	7.90

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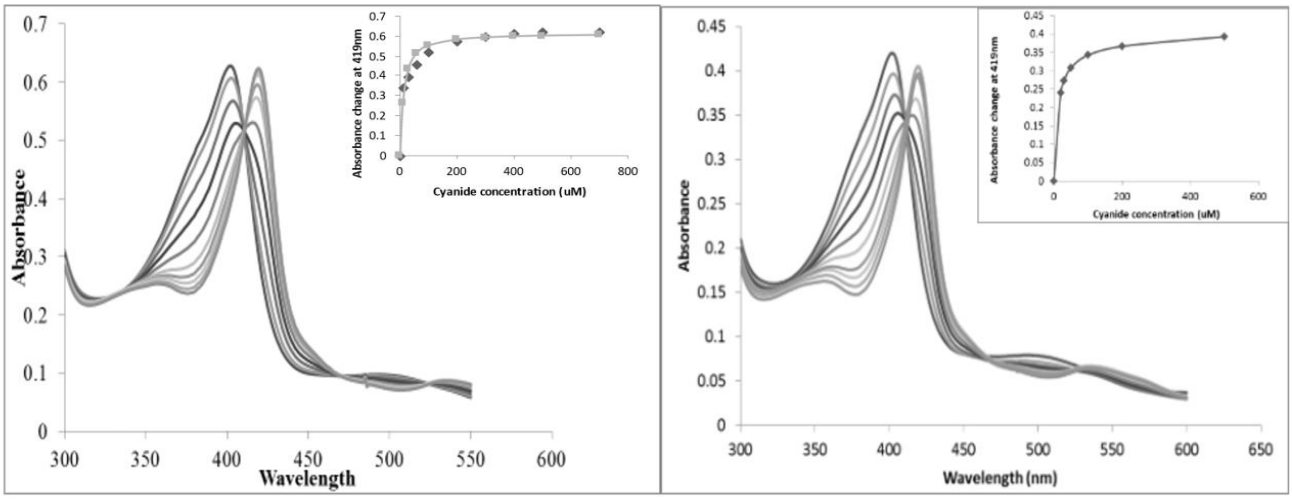

Figure 5: Ligand binding studies of (a) KSV8 and (b)SK5912 sorghum peroxidases with cyanide. Insert: Plot of the absorbance (extinction) changes at 419nm resulting from the binding of varying levels of cyanide to the sorghum peroxidases

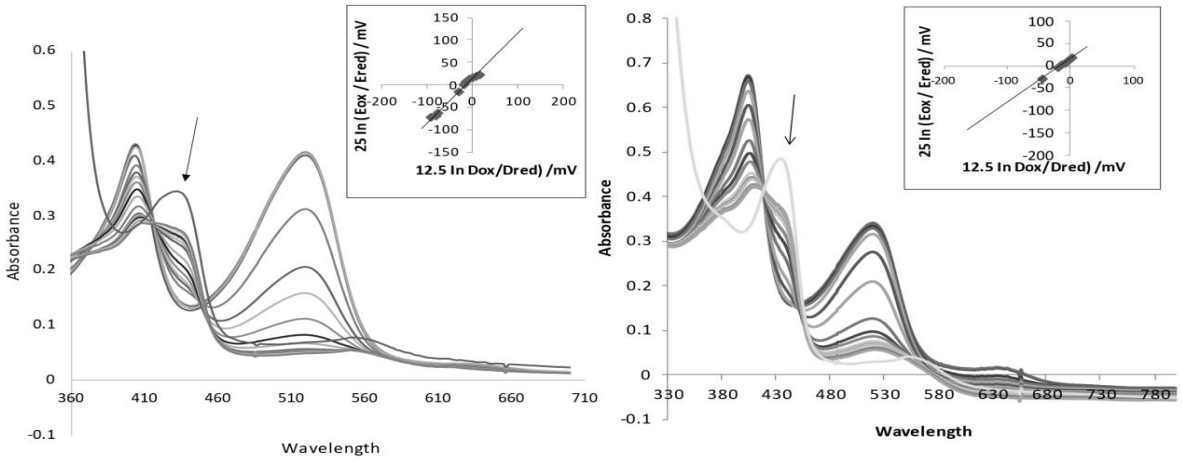

Figure 6: Reduction potential reaction of (a) KSV8 and (b) SK5912 sorghum peroxidase with phenosafranin. The spectrum indicated with the arrow showed full reduction after addition of dithionite. Inset: Nernst plot of the reduction potential of sk 5912 sorghum peroxidase with phenosafranin

Table 3a: effect of metal ions and some chemical inhibitors on the activities of KSV sorghum peroxidase

Enzyme + metal or chemical	Reaction rate	Relative activity	Fold change	Effect on peroxidase activity
Enzyme alone	0.529 ± 0.02	100	1	Control
Enzyme + Ca^{2+}	6.028 ± 0.05	1140	11.4	Huge increase
Enzyme +Mg^{2+}	0.638 ± 0.01	121	1.21	Slight increase
Enzyme + Cu^{2+}	0.365 ± 0.02	69	0.69	Decrease
Enzyme + Fe^{3+}	6.15 ± 0.41	1163	11.6	Huge increase
Enzyme + Co^{2+}	0.405 ± 0.02	77	0.77	Decrease
Enzyme + Na^{+}	0.175 ± 0.03	33	0.33	Huge decrease
Enzyme + K^{+}	0.345 ± 0.03	65	0.65	Decrease
Enzyme + NH_4^{+}	0.32 ± 0.02	61	0.61	Decrease
Enzyme + Rb^{+}	0.306 ± 0.04	58	0.58	Decrease
Enzyme + Ferricyanide	0.175 ± 0.01	33	0.33	Huge decrease
Enzyme + EDTA	0.559 ± 0.04	16	1.06	Slight increase
Enzyme + DTPA	0.891 ± 0.03	168	1.68	Increase

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Table 3a: Effect of metal ions and some chemical inhibitors on the activities of SK5912 sorghum peroxidase

Enzyme + metal or chemical	Reaction rate	Relative activity	Fold change	Effect on peroxidase activity
Enzyme alone	0.232 ± 0.011	100	1	Control
Enzyme + Ca^{2+}	8.251 ± 0.132	3560	36.5	Huge increase
Enzyme +Mg^{2+}	0.351 ± 0.015	151	1.5	Slight increase
Enzyme + Cu^{2+}	1.565 ± 0.211	675	6,75	Increase
Enzyme + Fe^{3+}	0.532 ± 0.056	230	2.3	Increase
Enzyme + Co^{2+}	0.333 ± 0.022	144	1.44	Slight increase
Enzyme + Na^{+}	0.171 ± 0.018	0.74	0.74	Decrease
Enzyme + K^{+}	0.232 ± 0.008	100	1	No change
Enzyme + NH_4^{+}	0.221 ± 0.013	95	0.95	Slight decrease
Enzyme + Rb^{+}	0.176 ± 0.061	76	0.76	Decrease
Enzyme + Ferricyanide	0.281 ± 0.013	121	121	Huge Increase
Enzyme + EDTA	0.200 ± 0.002	86	0.86	Decrease
Enzyme + DTPA	0.280 ± 0.031	121	121	Slight increase

Conclusion

In this study we demonstrated a simple few-step protocol for purifying peroxidases from two sorghum varieties, KSV8 and SK5912, and compared some of their key properties. Our findings show that both peroxidases share a few of their properties but differ in many. Such comparative works help to elucidate the key differences between different cereal grain varieties especially among many of the newly improved ones that are important and useful in many industrial and biotechnological applications. These experiments with sorghum are important because apart from the many cultivation advantages resulting from its use, such as its high drought tolerance, and other important roles it plays in food and agriculture as natural products, results obtained from them are useful in determining their suitability for use in the food, chemical and allied industries.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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