ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Changes in the activities of peroxidases during different stages of sorghum malting

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Manuscript Info

Manuscript History:

Received: 14 August 2013
Final Accepted: 25 August 2013
Published Online: September 2013

Key words:
Peroxidase, enzyme, malting, brewing, sorghum, cereals.

Abstract

In this study changes in the peroxidase expression of ten different sorghum varieties were monitored during a 96-hour malting period in order to determine and track how the expression of the enzyme is affected by malting. Results obtained showed that the different sorghum varieties differed in their expression of peroxidase and that this difference was also reflected across the different malting regimes. While the least expression of peroxidase, which stood at ≤ 0.6 peroxidase units across all the varieties, was observed at the end of steeping, the highest peroxidase activity was observed to occur between the 72^{nd} to 96^{th} hours of germination. These highest peroxidase levels which stood at above 6 peroxidase units were obtained consistently with varieties SK5912, KSV8, White kaura, Boboje, NRL and also with ICSV III. To some extent, the size of the sorghum grains affected their peroxidase expression. Considering the many important and multi-faceted roles that peroxidases are nowadays known to perform, the study of their expression dynamics in different plants and processes, one of which we report here, could help further the understanding of their characteristics and thus facilitate their maximal utilization. The increasing importance of sorghum due to its many physiologically advantageous attributes and the multiplicity of available varieties justify its use in the study.

Introduction

Plant peroxidases (EC 1.11.1.7) are heme-proteins that use H_2O_2 to oxidise a large variety of hydrogen donors such as phenolic substances, amines, ascorbic acid, indole and certain inorganic ions (Dunford, 2010; Murphy et al., 2012). These enzymes occur widely in animals, plants and microorganisms, where their repertoire of activities include catalytic, hydroxylation and oxidative reactions (Dunford, 1999; Diao et al., 2011). In the plant kingdom peroxidase and her different isoenzymes are known to occur in a variety of plant types and tissues such as grains and cereals. Indeed many grains such as barley, wheat, buckwheat, soybean etc. have been shown to exhibit high levels of peroxidase activities (Zmrhal and Machackova, 1978; Sessa and Anderson, 1981; Clarkson et al., 1992; Suzuki et al., 2006). In the plants where they occur, peroxidases play many roles but mainly serve to catalyze the reductive destruction of hydrogen peroxide, which otherwise could lead to lipid peroxidation (Nwanguma and Eze, 1995). In most cereals lipid peroxidation causes reduction in quality and shelf life of the products (Hilderbrand, 1992). Beer is one food item whose flavour is deteriorated due to lipid oxidation during production (Dey et al., 2005). In the brewing process, especially during the malting stage, the release of aldehydes and other lipid peroxidation products have been shown to affect the availability of wort nutrients, interfere with yeast metabolism, as well as participate in reactions that affect the flavour and colloidal stability of beer (Bamforth et al., 1993). Peroxidases and other similarly acting enzymes such as catalase and superoxide dismutase are associated with the containment of these problems. The activities of peroxidases have been reported in different sorghum varieties (Nwanguma and Eze, 1995; Dicko et al., 2006). Sorghum, the fifth most important cereal in total world production, after

wheat, rice, corn and barley is extensively grown in different parts of the world with the US, Nigeria, India, China, Argentina, Mexico and Australia being the highest producers of the cereal (Wong et al. 2009; USDA, 2011). It is used for different food and industrial purposes with several types of indigenous foods, and also alcoholic and non-alcoholic beverages being produced using it (Owuama 1991; Asiedu 1992; Agu and Palmer 1998; Taylor et al. 2006). The reason for this extensive usage derives from the advantage sorghum has of being physiologically robust and able to withstand extreme environmental circumstances such as extreme drought and heat as well as temporary water-logging among others (Purselove 1972; Taylor et al. 2006; Ejeta and Knoll, 2007). Nwanguma and Eze (1995) had previously done some work on how peroxidase activities change during the course of sorghum malting. In their work however, they had used only two varieties of sorghum. For greater representation and breath, we have therefore used more varieties in this work, including the two they had used to serve as some sort of reference. Additionally unlike in their work, greater depth and attention to how each of the different stages affects peroxidase during malting is dealt with more comprehensively in the present study. The choice of peroxidase activity as the subject of our inquiry in this work stems from different fronts. On the one hand is the fact that it is often considered as a representative antioxidant enzyme (Lin et al., 2008). Further to that is its relative ease of assay and reproducibility, as well as the established importance of peroxidases. Peroxidases have been reported to have immense biotechnological applications most of which arises from the different chemical reactions it catalyzes (Colonna et al. 1999). Examples of these applications are seen in their use in the treatment of industrial waste waters; synthetic organic chemistry to catalyze different fine chemical processes; as an important enzyme for diagnostic test kits; in lignin biodegradation and in the modification of carbohydrate-containing hydroxycinnamates (Hamid and Khalil-ur-Rehman, 2009). All of these biotechnological possibilities and potentials make the study of peroxidases of evergreen interest. These promises extend to sorghum considering its diverse properties and importance. We therefore, in this work, report the changes in the peroxidase expression of ten different sorghum varieties monitored over a 96-hour germination period.

Material and Methods

Grain sourcing and cleaning

Ten different varieties of sorghum grains (*Sorghum bicolor* L. Moench var. SK 5912, KSV 8, ICSV 400, ICSV III, White Kaura, Boboje, CSRO2, NRL-3, KAT 487 and Nafelen 6) all grown in 2009 and purchased from the Institute for Agricultural Research of the Ahmadu Bello University Zaria, Nigeria were used for this work. The method of Ogbonna et al. (2003) was used to clean and sort the grains.

Thousand kernel weight (TKW) determination

The thousand kernels weight, a measure the relative size of the sorghum grains, was done by manually counting out one thousand pieces of the sorghum grain and weighing them afterwards (Bamforth, 2002).

Grain steeping

Sorghum grain steeping was done by measuring out exactly 200 grams of each sorghum variety in triplicates, and immersing them in 400 ml of distilled water such that the grain/water mixture was in a ratio of 1:2. Steeping was carried out for 24 hours at room temperature (28^{o}C). The steep water was changed at 6 hourly intervals to remove any untoward microbial contaminant.

Germination

Sorghum grains were germinated for 4 days as described by Ogbonna et al. (2003). After germination the grains were immediately freeze-stored at -80^{o}C for at least 24 hours or until required for assay. Before assay, the very cold and now brittle radicles and plumules were carefully removed manually.

Assay for Peroxidase Activity

Peroxidase was isolated using the method of Mclellan and Robinson (1981). After germination and freeze-storage as stated above, sorghum grains were then ground with a blender (James Martin, made in England) and then extracted by incubating the ground grains in a 1:2 (w/v) ratio with 0.1 M sodium phosphate buffer, pH 6.0 for 30 minutes at 4^{o}C. Afterwards, the extract suspension was centrifuged at 5000g for 30 minutes in a SS 34 Sorvall Evolution centrifuge (Made in USA) at 4^{o}C for 30 minutes before being filtered through a double-layered cheese cloth. Peroxidase activity was determined by measuring the change in absorbance at 470nm of guaiacol (Sigma chemicals co.) as it is oxidized to tetraguaiacol by the enzyme. The final reaction mixture contained 15 µl crude enzyme concentration, 1.5mM (15 µl of 100mM) guaiacol, 0.5mM (10 µl of 50mM) H_2O_2 and 0.96 ml of 100mM sodium phosphate buffer, pH 6.0 (making a total of 1 ml). The assay was performed at 25^{o}C using a UV-Vis scanning spectrophotometer (Perkin Elmer Lambda

35) with temperature control. The spectrophotometer is linked to a Graphics series (G70fmb) Dell computer. The increase in absorbance at 470 nm was monitored for 90 seconds with the slope of the linear initial portion of the curve used to determine activity. Enzyme activity was calculated using an extinction coefficient of 22.6 mM^{-1} cm^{-1} for tetraguaiacol (Santimone, 1975). One unit of enzyme activity was defined as the amount of enzyme that causes an absorbance change at 470 nm of 0.1 in one minute.

Results

Figure I show the relative size of the ten sorghum varieties used in this work. The highest sized grains used were SK5912, NRL3 and also KSV8, while among the lowest sized ones were Nafelen 6, KAT487 and ICSV III. As can be observed from the next figure, each of the sorghum varieties already contains peroxidase although these differ in amounts among the varieties. The highest peroxidase activities shown in the raw, unmalted sorghum varieties (figure 2) are from varieties SK5912, Boboje and white kaura. Some of these are among the biggest sized sorghum grains. On the other hand, the least peroxidase-containing varieties included Nafelen 6, KAT 487 and ICSV III, which incidentally are among the least sized grains. In figure 3, a general decrease in the activity of the peroxidases across the different varieties occurred. These were very significant decreases reaching over 100% in some varieties (Boboje, SK5912 and White kaura) from their values before steeping. On the other hand, the decrease was not so high among some of the other grains although most of them had activity reductions above 50%.

Figure 4 shows the peroxidase activity of the different sorghum varieties after they had germinated for 24 hours. Here the enzyme activity had picked up again from the initial decrease after steeping, although they had not reached the levels they showed in the raw grains. Again the grains, SK5912, KSV 8 and white kaura showed greatest increases at this initial germination period. Higher levels of peroxidase activity over the control (raw grains) were also obtained after the sorghum grains had germinated for 48 hours as shown in figure 5. The activity of the sorghum grain varieties also increased further after they had been germinated for 72 hours (figure 6). As a matter of fact, some of the grains had by this time reached the peak of their peroxidase activity. For example, variety SK5912 had at this point, reached its maximum activity value of about 6 peroxidase units, as was also the case with variety KSV 8. Figure 7 shows the peroxidase activity values of the different sorghum varieties after 96 hours of germination. At this point, most of the smaller sized grains had reached their maximal level of activity.

However, a much clearer perspective on the results are obtained when each of the grains is taken singly over the six treatment schedules of raw, steeped, 24h germination, 48h germination, 72h germination and 96h germination. For easy comparisons, these are shown in figures 8 to 17 for the ten sorghum grain varieties. A lot of things become obvious then. Taking variety ICSV 400 (figure 8) alone over the 96 hour period for example, it is seen that malting did not very significantly alter the peroxidase content of this variety from what was originally contained in the unmalted sorghum as only a difference of about 15% existed between them after malting for 96 hours. Such low differences in peroxidase expression before and after malting were not seen in the same low magnitude with other sorghum varieties. For example, the next lower differences between the raw sorghum (control) and after the highest peroxidase activities, achieved at least after 72 hours of germination, were given by Boboje (figure 12), White kaura (figure 11) and SK5912 (figure 9), which were 68%, 90% and 94% respectively. Most of the other varieties however showed somewhat similar positive results, which were considerably greater increases in the elaboration of peroxidase after malting than these already mentioned types. For example, a difference of almost 2-fold (194%) was observed between the raw sorghum grain (control) and the highest, after 72 hours of malting with variety KSV8 (figure 10), while for varieties KAT 487, NRL 3 and CSRO2 (figures 16, 15 and 14), the differences obtained were nearly 3-fold at 234%, 260% and 298% respectively, all of which were achieved after 96 hours of germination. Particular mention will however be made of varieties Nafelen 6 and ICSV III whose course showed the high impact malting had on some grains as their peroxidase activity showed increases of nearly 5-folds and 7-folds (444% and 678% respectively) from the values in the unmalted grains to that obtained after germination. A very interesting observation is the fact that most of the sorghum varieties that showed considerably great differences between the raw grains (controls) and at the end of germination, in their expression of peroxidase, such as ICSV III, Nafelen 6, KAT 487 were among the smallest sized varieties.

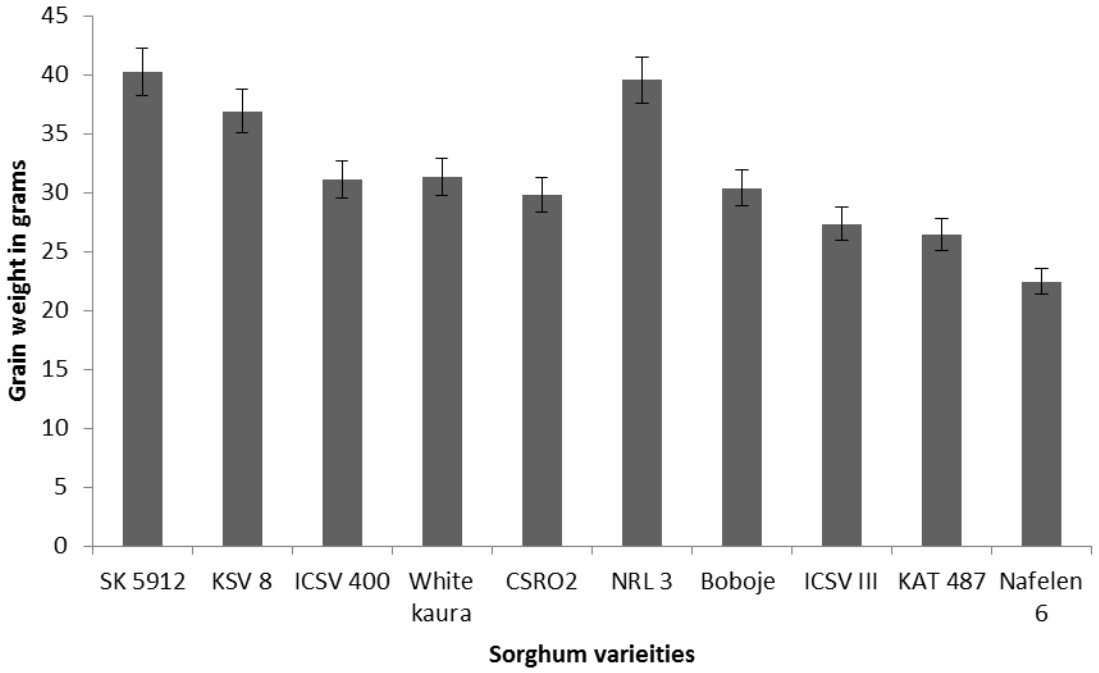

Fig 1 Thousand Kernel Weight (TKW) of sorghum grains

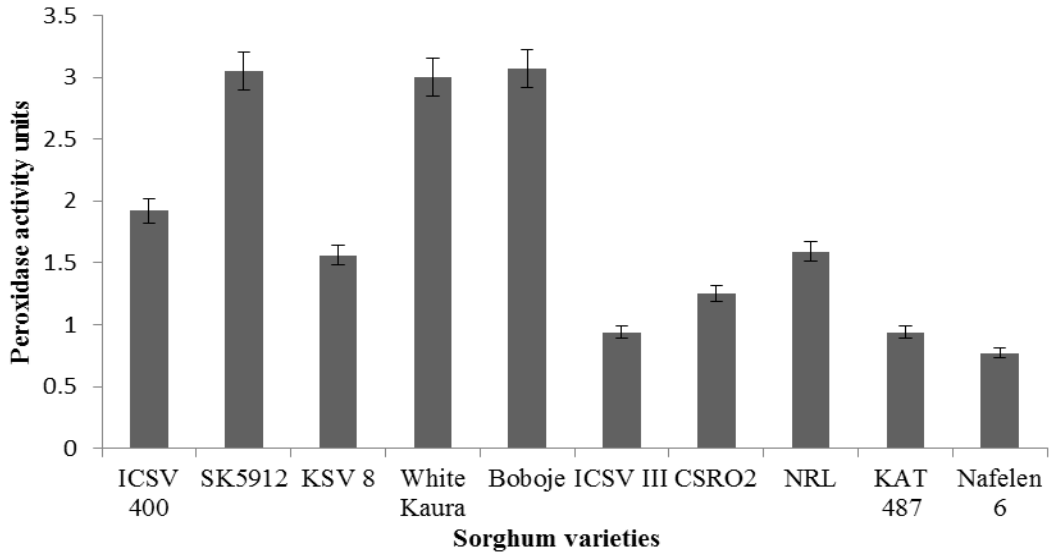

Fig 2 Peroxidase activity of raw grain sorghum varieties

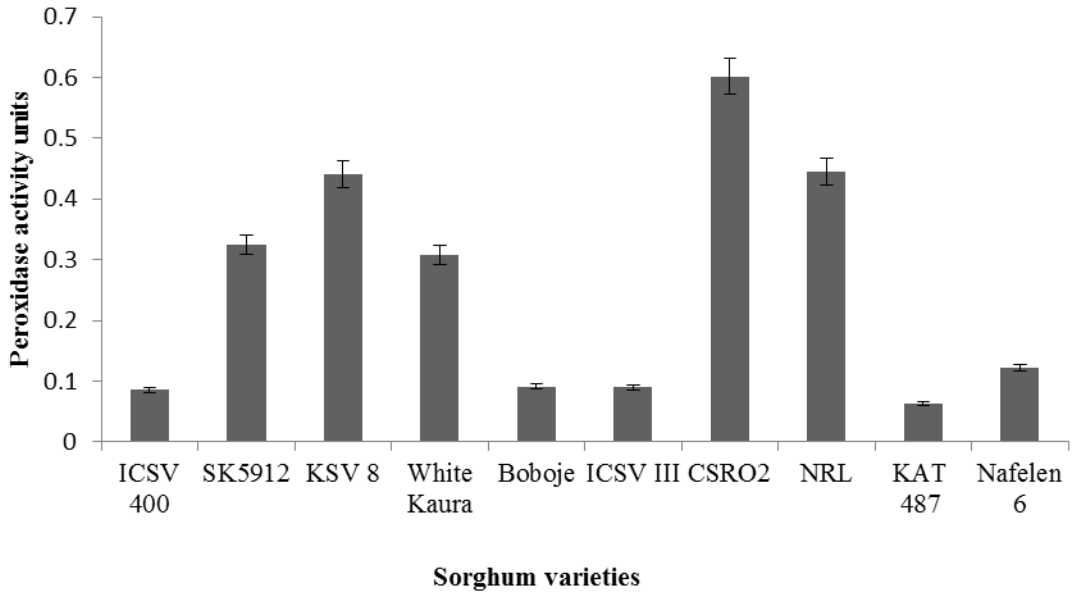

Fig 3 Peroxidase activity of sorghum grain varieties after steeping

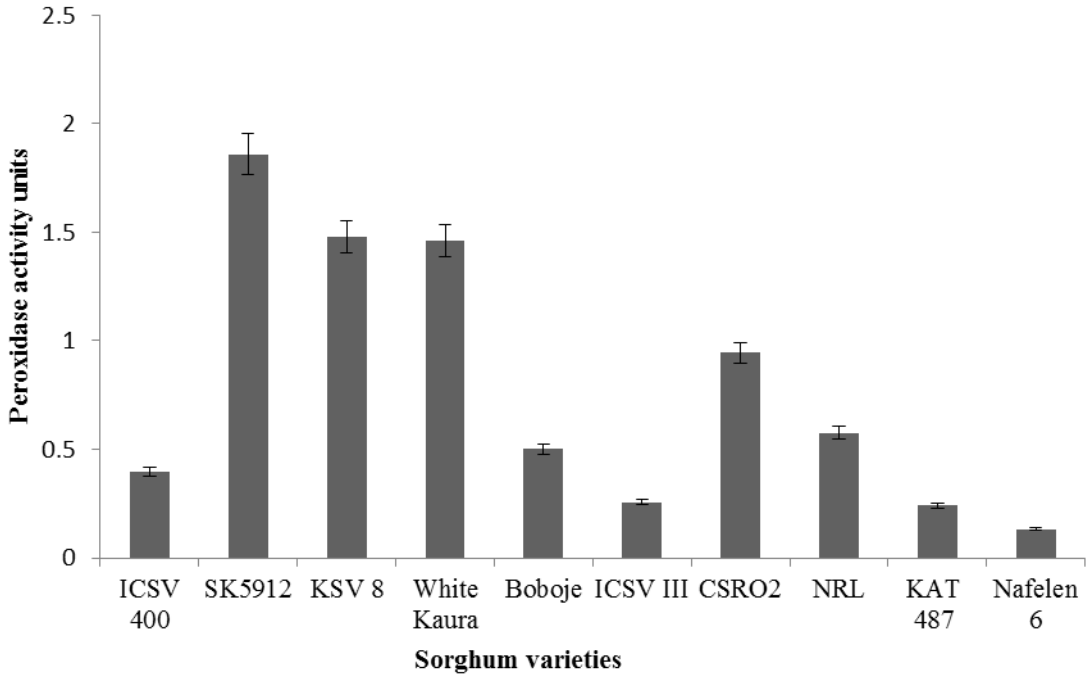

Fig 4 Peroxidase activity of sorghum grain varieties after 24h germination

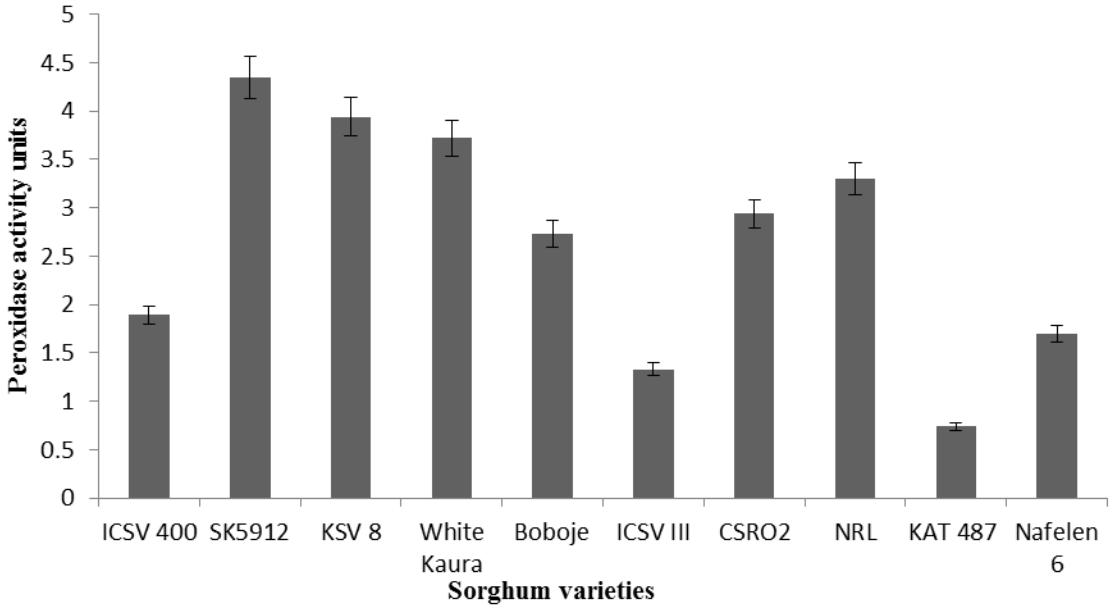

Fig 5 Peroxidase activity of sorghum grain varieties after 48h germination

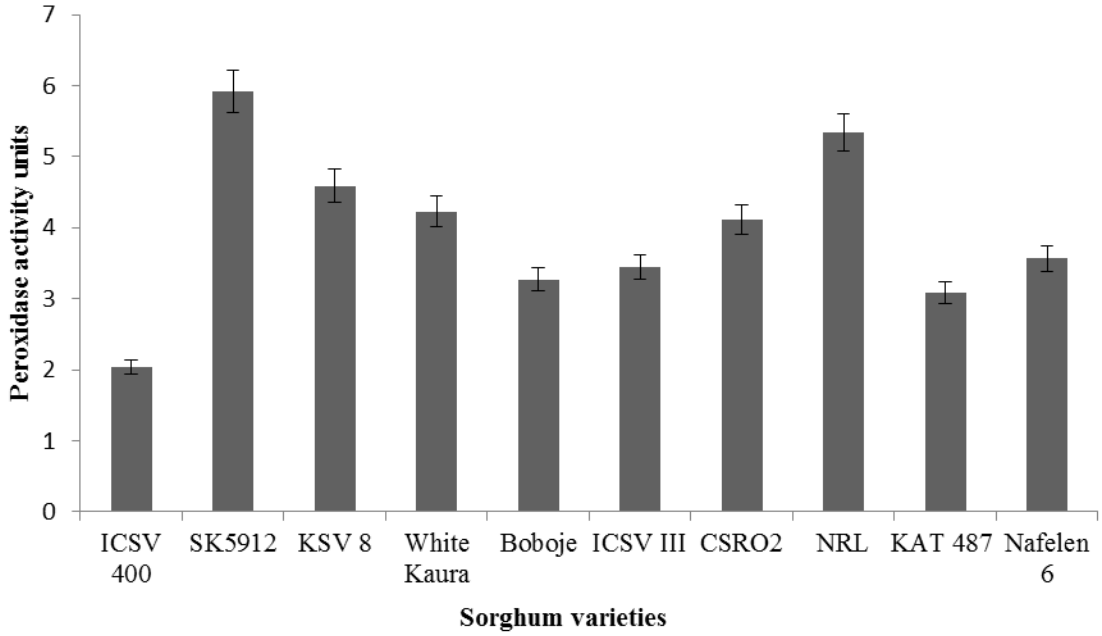

Fig 6 Peroxidase activity of sorghum grain varieties after 72h germination

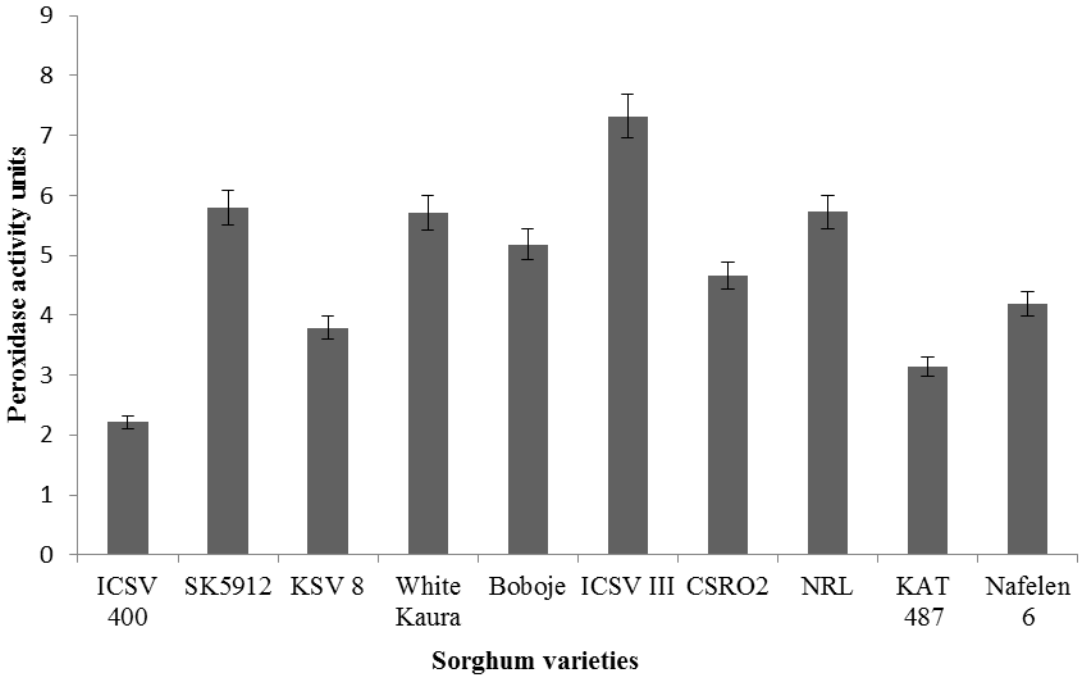

Fig 7 Peroxidase activity of sorghum grain varieties after 96h germination

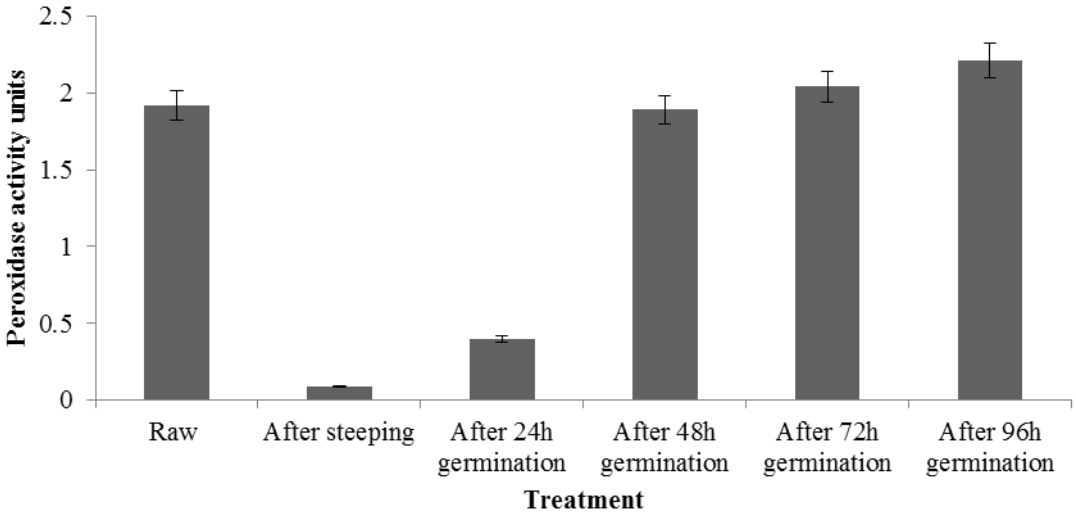

Fig 8 Changes in peroxidase activity of ICSV 400 after different malting stages

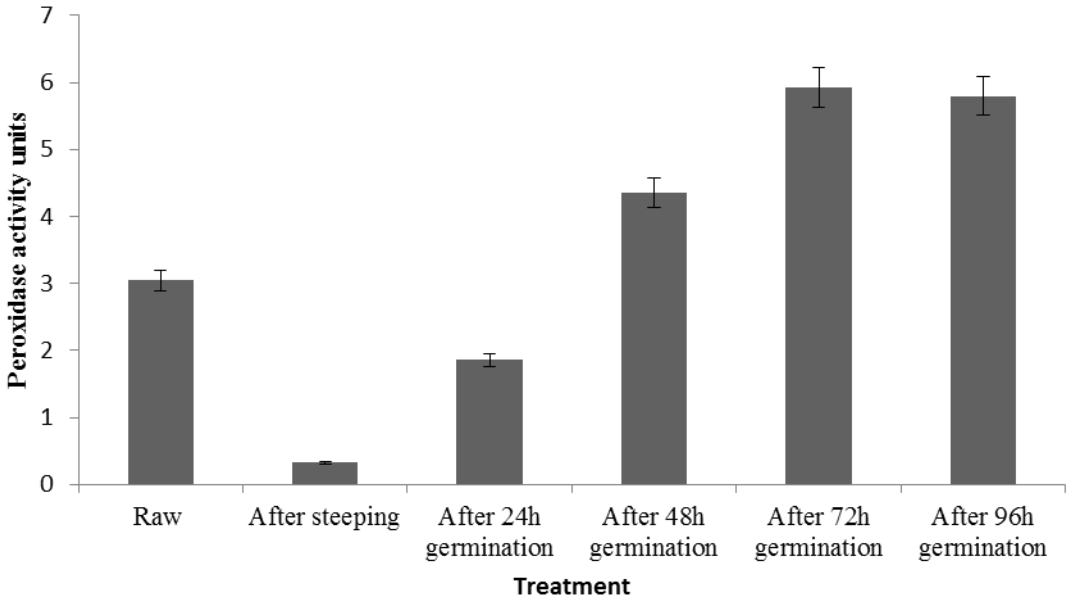

Fig 9 Changes in peroxidase activity of SK5912 after different malting stages

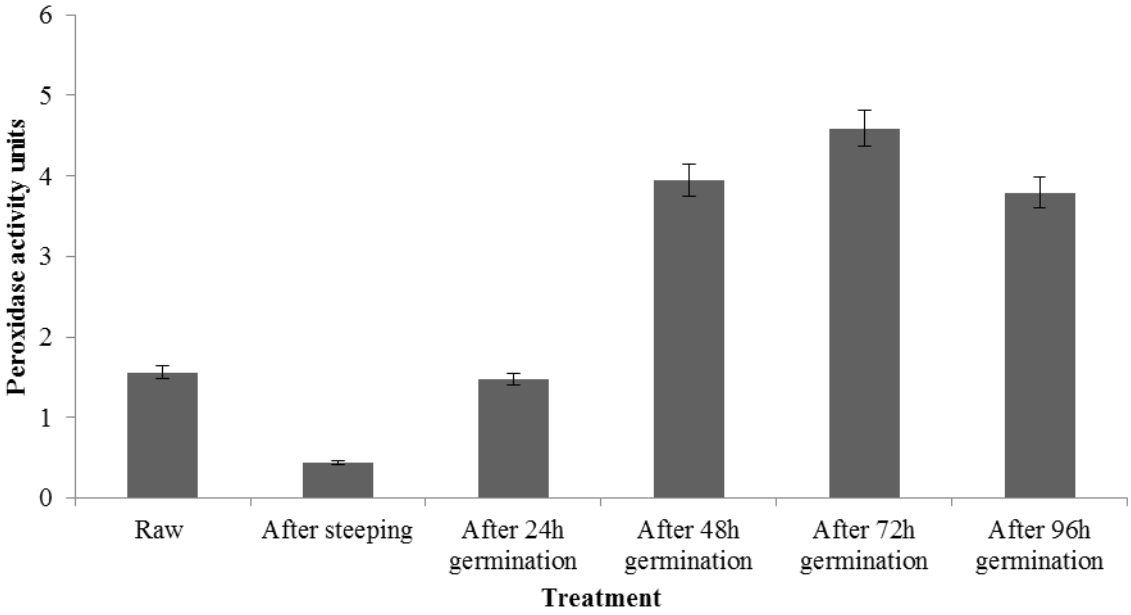

Fig 10 Changes in peroxidase activity of KSV 8 after different malting stages

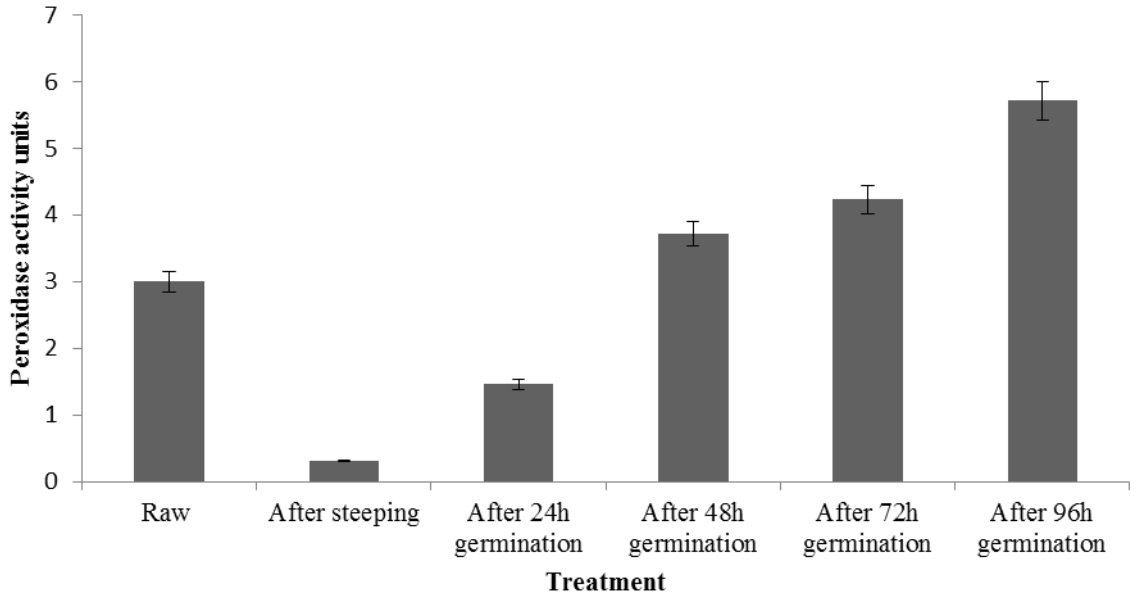

Fig 11 Changes in peroxidase activity of white kaura after different malting stages

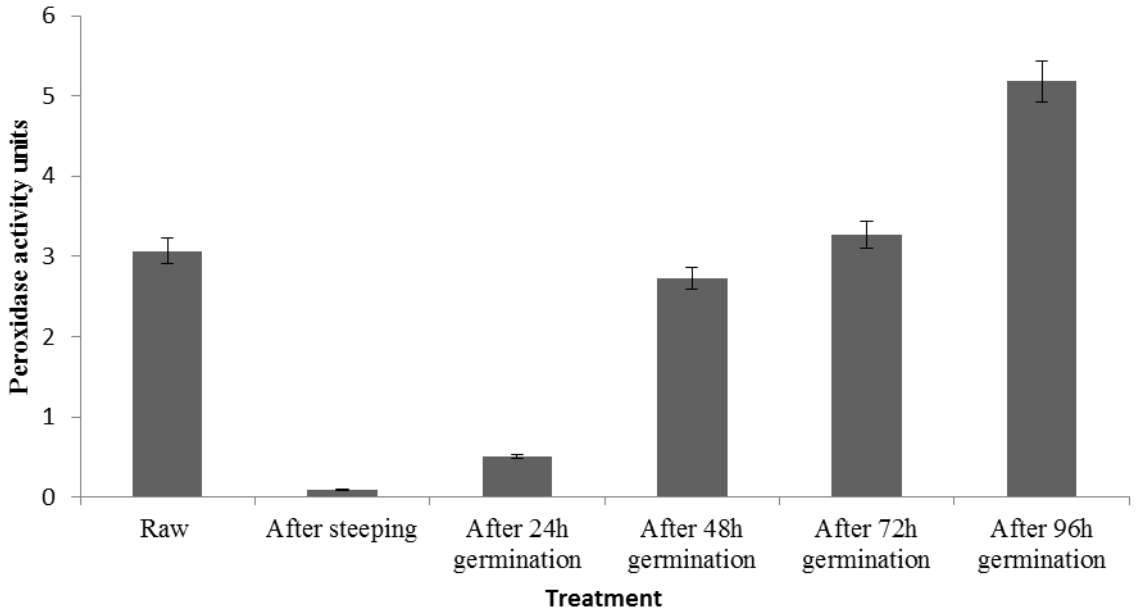

Fig 12 Changes in peroxidase activity of Boboje after different malting stages

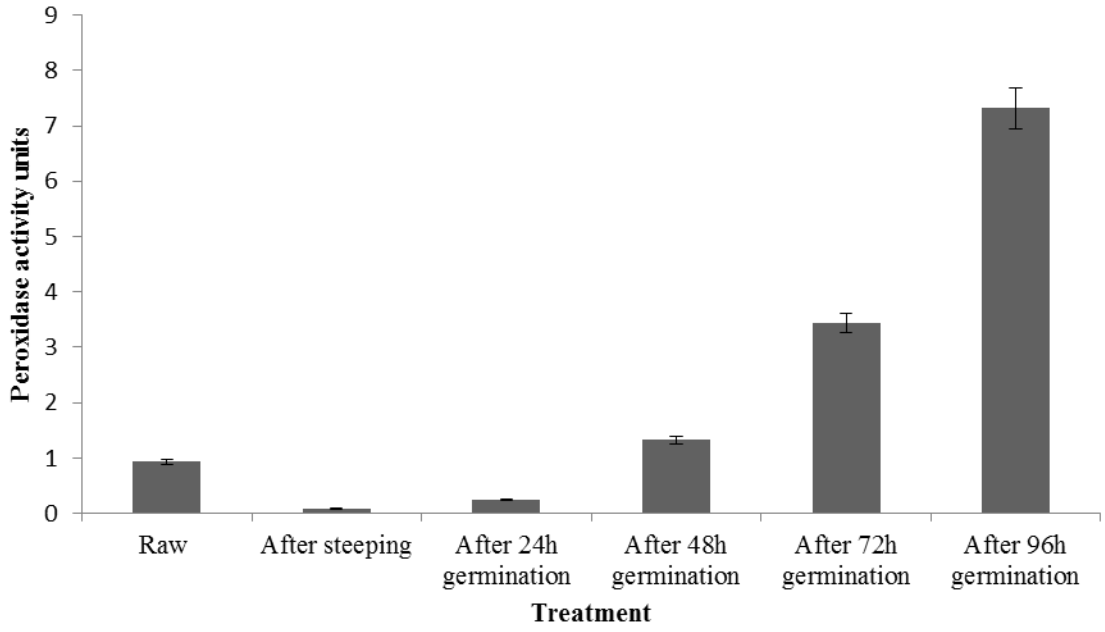

Fig 13 Changes in peroxidase activity of ICSV III after different malting stages

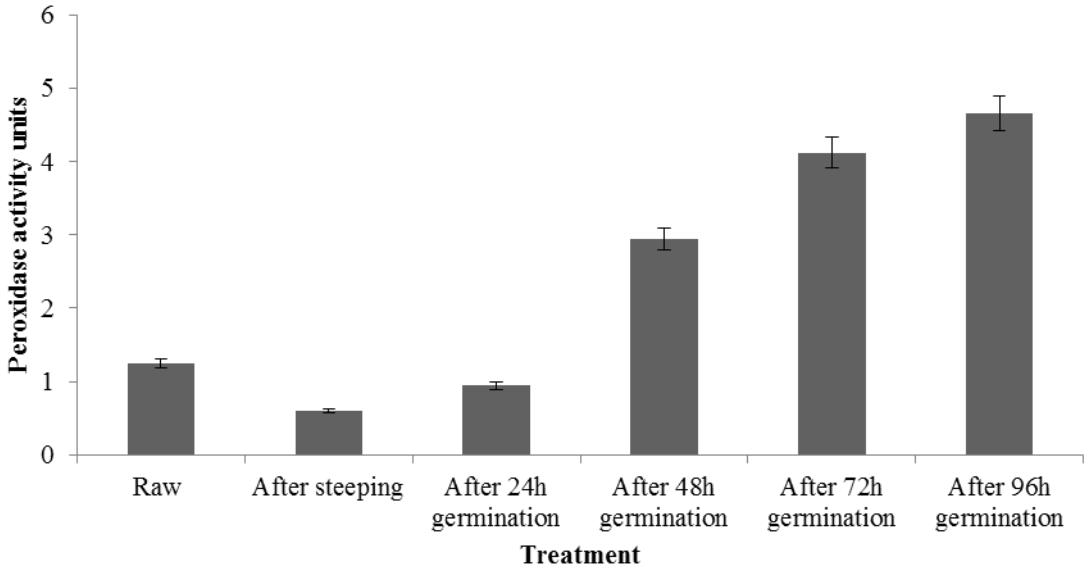

Fig 14 Changes in peroxidase activity of CSRO2 after different malting stages

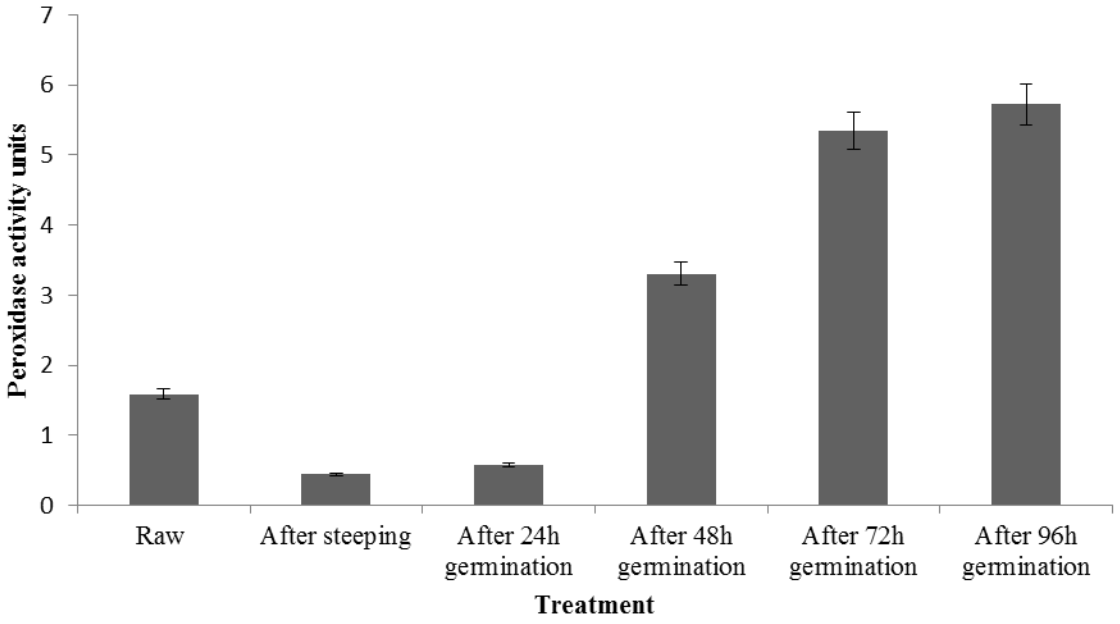

Fig 15 Changes in peroxidase activity of NRL after different malting stages

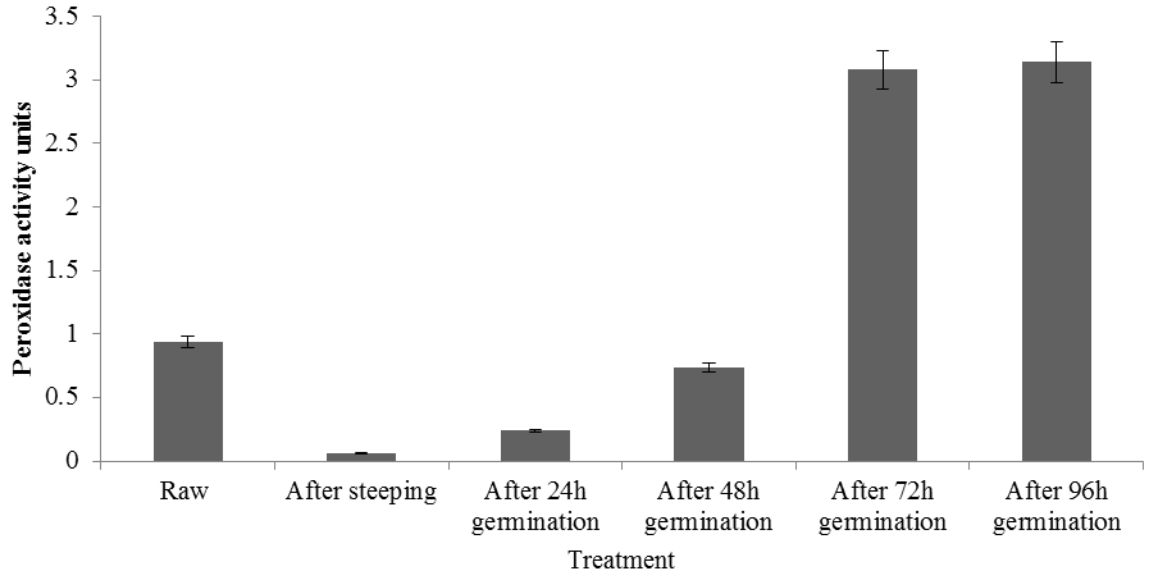

Fig 16 Changes in peroxidase activity of KAT 487 after different malting stages

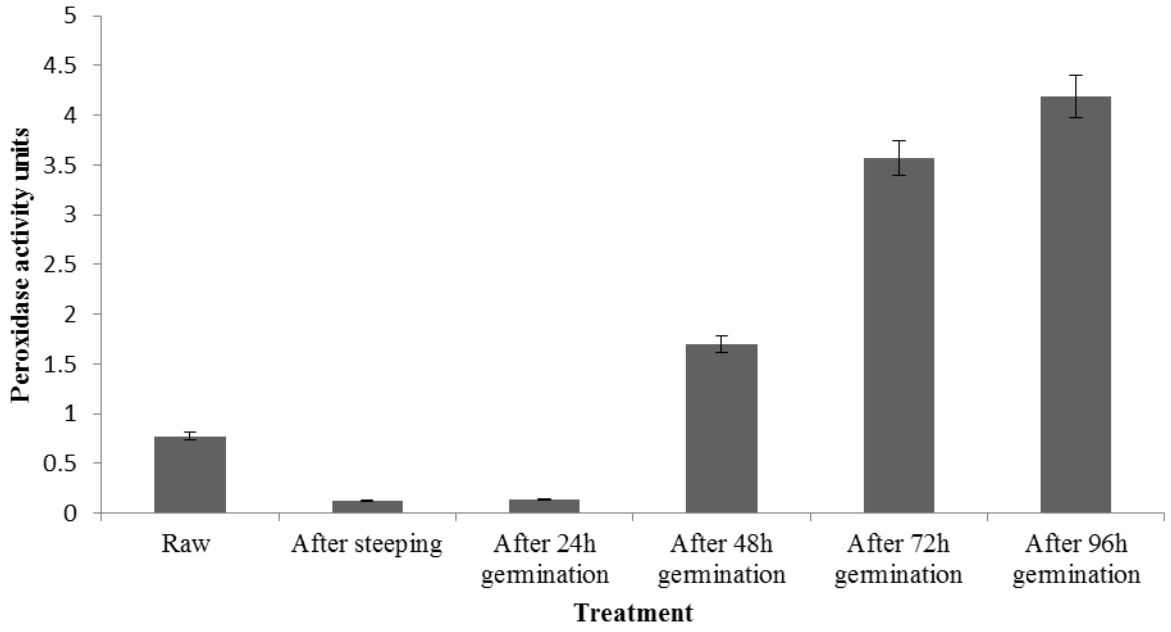

Fig 17 Changes in peroxidase activity of Nafelen 6 after different malting stages

Discussion

The presence of many antioxidant enzymes including peroxidases, catalases and superoxide dismutase has been demonstrated in many important cereals (Kruger, 1977; Nwanguma and Eze, 1995; Bakalova et al., 2004; Dicko et al., 2006; Ishibashi et al., 2008). These antioxidant enzymes function mainly to contain the excesses of the many free radicals generated during the many metabolic activities of the plants or organisms concerned, by reducing the energy of the free radical groups, donating electrons to them which then help to stabilize the free radicals by forming stable products or also to stop the formation of the free radicals in the first place. They may also interrupt an oxidizing chain reaction in order to minimize the damage caused by free radicals (Elliot, 2006). Although as stated above, the incidence of peroxidases and other antioxidant enzymes have been demonstrated in several cereals, only very few works had been done on their incidence using different sorghum varieties found in most parts of the world. We have therefore tried in the present dispensation to work with more improved sorghum varieties. As can be seen from the figures, the different malting processes greatly impacted on the peroxidase activities of the ten species of sorghum used here. One key observation is the fact that in all cases steeped grains elaborated the least expression of the enzyme as expressed in peroxidase activity; with values many times lower than those of the control (raw grain). It is as if steeping in some ways suppresses the expression and thus activities of the peroxidases. Steeping, which simply means the soaking of grains in water, is known to be a key factor, in short as the most critical stage in the malting process of cereals (French and McRuer, 1990). The importance of steeping lies in it being the point of initiation of germination which thereupon induces the activity of endosperm modification enzymes especially amylases, proteases and similarly working enzymes. These enzymes and their activities then ultimately determines how the cereal endosperm modification will occur especially as it concerns the production of the desired malt (Dewar et al., 1997). Because it is the point of initiation, the expression of the activities of many enzymes including peroxidases should therefore necessarily be at its lowest levels after steeping prior to germination. The consistency of that low expression as seen all through the different cultivars of sorghum grains used in this work somewhat establishes steeping as the point of lowest peroxidase expression among sorghum grains. A key observation to be noted here is the fact that whereas in the work of Nwanguma and Eze (1995),

the least peroxidase activity was obtained from the raw grains, it occurred here in all cases (including in the two they had used previously) after the grains had been steeped. This point is further established by the fact that the next stage of low peroxidase expression after steeping is again in all cases after the first 24 hours of germination. At that point the expression of enzymes like peroxidases, which were just initiated during steeping, has become increasingly more pronounced. The height of expression of peroxidase activity occurred after germination had progressed to many more 24-hour cycles up until the 96th hour of germination which is the last point after which germination is stopped. Therefore the development and expression of peroxidases in malting grains follows a linear relationship with increase in number and length of germination until a maximum expression point is reached beyond which further increase in enzyme activity may not occur. This point in the case of peroxidase malting under consideration here lies between the 72nd and 96th hours of germination because those were the points where the highest peroxidase activities occurred. Several authors had reported such increase in peroxidase expression with germination over time both with several cereals (Clarkson et al., 1992; Nwanguma and Eze 1995; Ishibashi et al., 2008). Several reasons have been given as responsible for the increase in peroxidase activity during the process of germination. The first may be related to their role in the growth process of seedlings, where it is said that peroxidases function in cell wall (lignin and suberin) biosynthesis, as well as in the metabolism of the plant growth hormone auxin (Passardi et al., 2004). Peroxidases are also believed to play protective and defensive roles (against pathogens and other enemies) in the life of germinating seeds as their expression immediately starts with the life of germinating seedlings usually in association with reactive oxygen species, ROS (Passardi et al., 2005). Scialabba et al. (2002) similarly reported the release of peroxidases and ROS during germination in the medium surrounding the seed in radish (*Raphanus sativa*). Additionally, the malting stage is believed to be the stage in brewing when lipid peroxidation as a result of the effect of the release of free radicals could best be controlled, therefore making it desirable and most appropriate that antioxidant enzymes like peroxidases could be expressed then (Nwanguma and Eze, 1995; Stephenson et al., 2003). Lipid peroxidation which refers to the oxidative degradation of lipids is akin to a process where free radical and ROS steal electrons from lipids in cell membranes resulting in cell damage. Furthermore, the high expression of peroxidases in germinated sorghum grains indicates that peroxidases are involved in the oxidation of endogenous phenolic compounds in sorghum flour during the processing of foods that require a germination step such as in beer, infant porridge and other beverages (Dicko et al., 2006). Also, the fact that the highest peroxidase activity sometimes occurred in the 72nd instead of the 96th hour as shown by many of the sorghum varieties here, is of significance considering that it could reduce the optimum time required for malting and thereby result in tremendous cost reduction in the different industrial production processes associated with it (Bekele et al., 2012). Finally, one notable point somewhat alluded to above, is the fact that smaller sized grains mostly had lower peroxidase activities in their raw state (controls) compared to their larger sized counterparts. However, by the end of malting, these showed significantly higher expressions of peroxidase than the larger sized varieties. Size may therefore, not necessarily give all the advantages, although when taken together, it makes sense to assume that the greater the grain size, the higher the activity of peroxidase expressed.

Conclusion

In this study, the effect of malting on the expression of peroxidase was studied using ten improved sorghum varieties. The different sorghum varieties showed varying levels of differences in how they were affected by malting. However, some common ground among all the grains were that steeping resulted in the least expressions of peroxidase activity across all the sorghum varieties, while the highest peroxidase activities were obtained after the grains had been germinated for at least 72 hours, meaning that malting impacted positively in the expression of peroxidase activities in sorghum as has been found also for other grain types. In addition, we also found that to a large extent, size of grains may have some effect on the expression of peroxidase activities in different ways: whereas larger sized grains tend to have high peroxidase activities right from the beginning of germination (between 24h to 48h), the smaller sized ones have more activities at the latter part of germination (between 72h to 96h). When taken together generally however, larger sized grains tend to have higher expressions of peroxidase activity than their smaller sized counterparts.

Acknowledgement

The authors wish to thank Nigerian government's Education Trust Fund (now Tertiary Education Fund, TETFund), for her grant to the first author which enabled him spend a year at the University of Leicester, England, where the work reported here was carried out.

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