Effect of Different Steeping Conditions on the Peroxidase Activities of Some Improved Sorghum Varieties

[1]Nnamchi C.I*., [2]Okolo, B. N., [3]Moneke, A. N. and [4]Nwanguma, B. C.
[1,2,3]Department of Microbiology, University of Nigeria, Nsukka;
[4]Department of Biochemistry, University of Nigeria, Nsukka;

Corresponding Author: Nnamchi C.I.; Phone: +2348037461157; Email: chukwudi.nnamchi@unn.edu.ng

Abstract
In order to evaluate the impact of duration and the incorporation or otherwise of air rest during steeping on the peroxidase activities of sorghum grains, five improved varieties were studied. Steeping durations used ranged from 0 (raw grains) to 72 hours, in two formats: with air rest and without air rest. Results obtained showed that among the raw grains which were the control experiment, variety SK5912 gave the highest peroxidase activity, followed by variety KSV 8 while variety CSRO2 had the least peroxidase activity. During steeping, we observed that in almost all cases, higher peroxidase activities were obtained with air rested sorghum grains than those without air rest. In terms of how the different varieties compared during steeping, we found that variety KSV 8 gave the highest peroxidase activity at both with and without air rest regimes in all the steeping periods used. Also, we observed that in all cases, the highest increase in peroxidase expression was obtained during the first 6 hours of steeping, shown by a steep rise of over 50% increase in activity. The other steeping times following the first 6 hours however showed just gradual increases in peroxidase over the period of sorghum. The major significance of this result is that although increasing duration showed increased peroxidase activities, such increases were less jumpy than those obtained during the first 6 hours. Therefore, long steeping times may not necessarily be very beneficial, with the added benefit that costs associated with long steeping durations may be avoided.

Key words: Sorghum, cereals, peroxidases, enzymes, steeping, germination, air rest.

Introduction

The steeping period is often considered one of the most important and critical parts of malting, a required process in most food and industrial based processes (French and McRuer, 1990; Igyor *et al*., 1989). Steeping generally involves the soaking of grains in water for some time, before the steeped grains will in the majority of cases be subjected to further treatment which is germination (Anderson, 2000). During steeping the grain swells by about 25% and softens as cell metabolism recommences. It is believed that water uptake is initially rapid but gradually plateaus out as steeping progresses. The rate of hydration of grains during steeping is said to be dependent upon variety, grain sample, corn size, nitrogen content, temperature among other factors (Briggs, 1998). As the immersion period progresses, the steep water becomes discoloured due to the presence of dissolved materials and microbes from the outer layers of the grain and is accordingly changed at least once (Anderson, 2000).

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 grain type plants such as barley, wheat, buckwheat, soybean, sorghum 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 beer production (Dey *et al*., 2005). In the brewing process, especially during the malting stage, the release of aldehydes, lipid peroxidation products, has 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. Frequently, peroxidases are considered as important representative antioxidant enzyme (Lin *et al*., 2008).

In a previous experiment, we found that steeping caused the least peroxidase expression in an experiment to assess the changes that occurred during the different stages involved in sorghum malting, including being lower in most cases than the amounts obtained with the raw sorghum grains. It was as if steeping in some ways suppresses the expression and thus activities of sorghum peroxidases (Nnamchi *et al*., 2013). Because the steep regime used in that experiment was a straight 24 hours of steeping with just a change of steep water every 6 hours, and no other treatment, we felt that the introduction of further alterations, such as

the introduction of air rest periods and also further increasing the length or duration of steeping time could produce better outcomes which will ultimately lead to higher quality of malted grains. Therefore, our aim in this work is to report the effects of these different steeping regimes in the peroxidase activities of different sorghum grains.

Materials and Methods

Grain sourcing and cleaning: Five different varieties of sorghum grains (*Sorghum bicolor* L. Moench var. SK 5912, KSV 8, ICSV 400, ICSV III and CSRO2) all grown in 2011 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 thereafter carried out in two ways, with or without air rest, for a total length of 72 hours at room temperature (28^{o}C). In that without air rest, the steep water was merely changed at each assay period in order to remove any untoward microbial contaminant, while in the other, an air rest period of 3 hours was introduced at the different assay intervals. Assay intervals during which readings were taken were at 6, 12, 24, 36, 48, 60 and 72 hour periods. Raw, un-steeped grains served as control.

Assay for Peroxidase Activity: Peroxidase was isolated using the method of Mclellan and Robinson (1981). After treating 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 room temperature. Afterwards, the extract suspensions were manually shaken for about an hour 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 Spectronic 21D (Milton Roy, Made in USA) UV-visible spectrophotometer. 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), and denoted as change in reaction rate. 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 and Discussion

Figure I shows the relative size of the ten sorghum varieties used in this work. The highest sized grains used were SK5912, and KSV8, while among the lower sized ones were varieties ICSV 400, ICSV III and CSRO2. As can be observed from the next figure, each of the sorghum varieties already contains peroxidase although these differ in amounts among the varieties. Again variety SK5912 had the highest expression of peroxidase, followed by KSV 8 while the least amount of peroxidase among the raw sorghum grain varieties was given by variety CSRO2 (as shown in Figure 2).

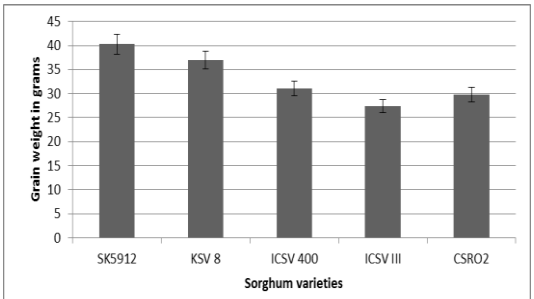

Figure 1: Thousand Kernel Weight (TKW) of sorghum grains

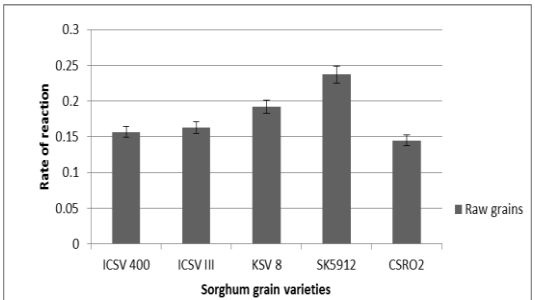

Figure 2: Peroxidase activities of raw sorghum grain varieties

Shown in Figure 3 is the peroxidase activity of the different sorghum grains after 6 hours of steeping. Here, the highest peroxidase activity was given by variety KSV 8, followed by variety ICSV 400, while the least enzyme expression was given by variety ICSV III, although its value was not so much different from those given by the other two varieties SK5912 and CSRO2. The figure also shows that the grains steeped with air rest in all cases gave higher peroxidase assay values. Similar result trends to the above (Figure 3) were also observed in figures 4 to 8, with variety KSV 8 elaborating the highest peroxidase activity all through, followed also by variety ICSV 400. However, in these other Figures (4 to 8), the gap in peroxidase expression levels between the varieties were closer than that observed in Figure 3. Again in

all of the varieties, grains steeped with air rest consistently gave higher peroxidase activities than their counterparts that were steeped without air rest.

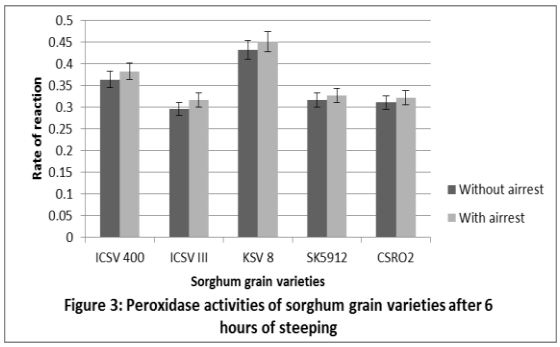

Figure 3: Peroxidase activities of sorghum grain varieties after 6 hours of steeping

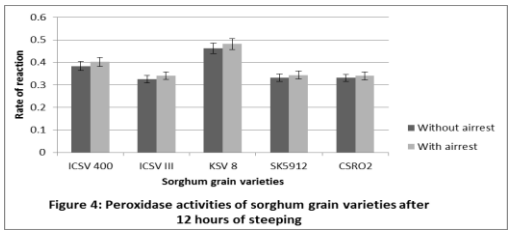

Figure 4: Peroxidase activities of sorghum grain varieties after 12 hours of steeping

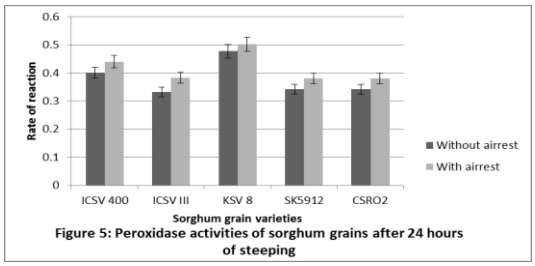

Figure 5: Peroxidase activities of sorghum grains after 24 hours of steeping

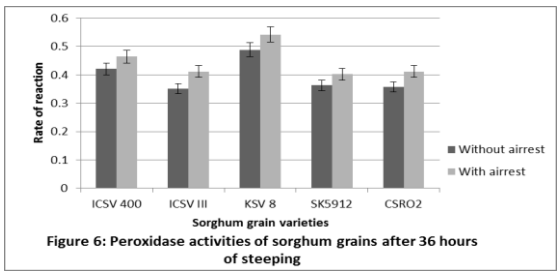

Figure 6: Peroxidase activities of sorghum grains after 36 hours of steeping

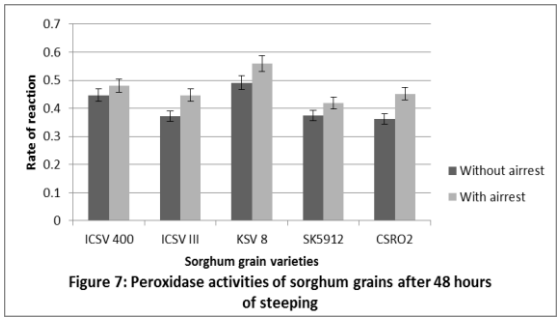

Figure 7: Peroxidase activities of sorghum grains after 48 hours of steeping

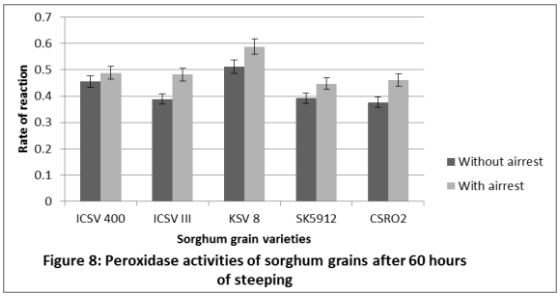

Figure 8: Peroxidase activities of sorghum grains after 60 hours of steeping

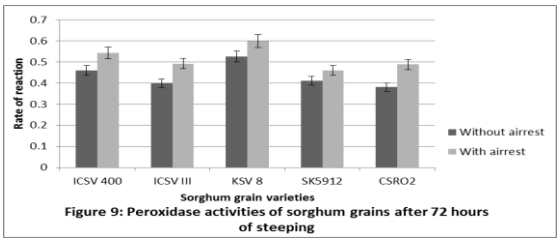

Figure 9: Peroxidase activities of sorghum grains after 72 hours of steeping

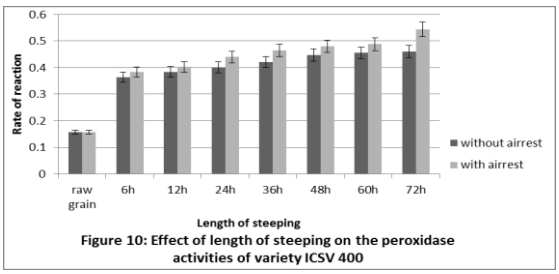

Figure 10: Effect of length of steeping on the peroxidase activities of variety ICSV 400

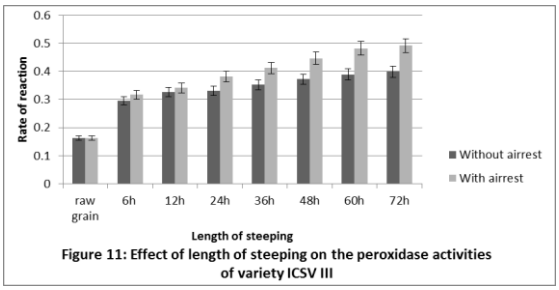

Figure 11: Effect of length of steeping on the peroxidase activities of variety ICSV III

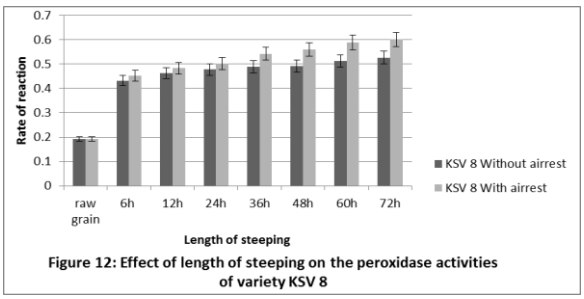

Figure 12: Effect of length of steeping on the peroxidase activities of variety KSV 8

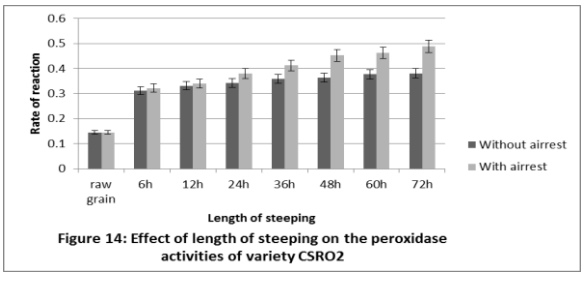

Figure 14: Effect of length of steeping on the peroxidase activities of variety CSRO2

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, 6, 12, 24, 36, 48, 60 and 72 hours of steeping. For easy comparisons, these are shown in figures 9 to 14 for the five sorghum grain varieties used in the study. A lot of things become obvious then. For example, it is seen that all the sorghum varieties exhibited the highest single increase in peroxidase expression after they had been steeped for 6 hours, while with further increases in steeping time, a very gradual but evenly distributed increases in peroxidase activities were observed for all the varieties assayed. However within these steeping periods, there was a difference in degree of peroxidase expression among the different sorghum varieties. In figure 10 for instance, while a difference of 140% and 130% was obtained between the raw grain of variety ICSV 400 and that steeped after 6 hours, only 90% and 80% were the values obtained for the same parameters in variety ICSV III (Figure 11). The values were even much less for variety SK5912 (Figure 13), with only 37% and 33% being the values obtained with that variety. In contrast, the only minor differences were observed between the different steeping periods.

Many antioxidant enzymes including peroxidases, catalases and superoxide dismutase has been demonstrated to be present 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 have as their primary roles the containment of 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. Also, they may 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 or no works at all, had been done on how steeping duration and air resting affect their incidence using different sorghum varieties found in most parts of the world. We have therefore tried in the present dispensation to close these observed gaps. As can be seen from the figures, the different steeping processes greatly impacted on the peroxidase activities of the five species of sorghum used here. One key observation is the fact that in all cases grains steeped after 6 hours elaborated the highest individual expression of the peroxidases, with values many times higher than those obtained with the control (raw grains). Over the whole length of steeping, only slow rate of increases were observed showing that the most significant periods in grain steeping could be the first few hours. This fact could therefore significantly lower the time and duration of steeping and by extension the associated cost during grain malting processes of which steeping is a vital component (Igyor *et al*., 1989; Ukwuru, 2010). No central connection could be found linking grain weights with peroxidase activities excepting the fact that the two heaviest varieties SK5912 and KSV 8 gave the highest peroxidase activities in different aspects: whereas SK5912 gave the highest value among the raw grains, but consequently expressed comparatively low values during the course of steeping, KSV 8 consistently gave higher peroxidase values during the entire steeping process.

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 the fact that it is 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 present work did not go on to germination to ascertain that fact. However, several workers had reported increased expression of peroxidases as malting periods increases (Nnamchi *et al*., 2013; Nwanguma and Eze, 1995). An important observation seen here is the similarity somewhat of our result with that observed by Nwanguma and Eze (1995) that the least peroxidase activity was got from the raw grains. The finding that grains subjected to periods of air rest gave higher peroxidase activities than those without air rest is similar to the findings of different authors such as Ezeogu and Okolo (1995) who showed that the incorporation of air rest periods improved many malting parameters of sorghum including root lengths, malting loss, diastatic power, and also the enzymes α- and β- amylases. Others had found that air resting during steeping of grains such as barley accelerated germination and also caused the production of workable malts from difficult barleys (Briggs, 1987).

Several reasons could be given as being responsible for the increases in peroxidase activities observed during steeping and other malting processes. 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). Similarly, Scialabba *et al*. (2002) had reported the release of peroxidases and ROS during germination in the medium surrounding the seed in radish (*Raphanus sativa*). Being directly involved with germination, these roles no doubt also apply to steeping.

The results obtained here reinforces the earlier statement that steeping to a large extent is dependent upon variety, grain sample, corn size, nitrogen content, temperature among other factors (Briggs, 1998). Although, there are lots of similarities in a lot of the results obtained here, critical differences observed in many of the cases can only be sufficiently explained by attributing them to varietal and grain size differences among many others.

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