African Journal of Food Science Vol. 5(4), pp. 227 – 231, April 2011
Available online http://www.academicjournals.org/ajfs
ISSN 1996-0794 ©2011 Academic Journals

Full Length Research Paper

Susceptibility of steeped and heat dried cowpea flour to fungal growth and aflatoxins production

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Accepted 11 March, 2011

In vitro **studies were carried out to determine the susceptibility of flours from steeped and heat dried cowpea to fungal growth and aflatoxins production. A 2 x 7 factorial experimental design with 2 levels of steeping time of cowpea grain in water (0 and 3 min steeping) and 7 levels of drying temperatures of the grains ranging from 35 to 120°C were used for flour production. Processed flours were used to prepare solid Cowpea Flours Agar media (CFA). Aflatoxigenic strain *(Aspergillus flavus*) was inoculated to these media and incubated at 30°C for 7 days. Yeast Extract Sucrose (YES) was used as control. Radial growth of colonies formed was measured and aflatoxins production was determined using High Performance Liquid Chromatography (HPLC). After incubation period, maximal growth of *A. flavus* colonies was observed in all CFA. The aflatoxin levels detected in CFA prepared with flour dried at 35-50°C ranged from 4.40 to 29.63 µg toxin /kg flour, while higher levels ranging from 27.45 to 75.00 µg toxin /kg flour were detected in CFA prepared with flours dried at 60 -120°C. The effect of steeping cowpea in water on aflatoxin production was dependent on drying temperatures. The average aflatoxin levels in CFA flours obtained from non steeped and steeped grains were 11.16 and 16.14 µg toxin /kg flour respectively and were statistically similar at *P= 0.05* at 35-50°C drying temperatures. It was concluded that drying steeped or non steeped cowpea at temperatures less than 60°C exposes cowpea flour to minimal risk of aflatoxin contamination.**

Key words: Cowpeas flour, aflatoxins, drying temperature.

INTRODUCTION

Cowpeas (*Vigna unguiculata* (L.) Walp.) is a cheap and valuable source of protein in many tropical countries. However the quality and safety of agricultural product are of major concern due to increasing occurrence of chronic diseases associated with consumption of contaminated staple crops by the aflatoxins in these regions (FAO, 2000; FDA, 2001). Fortunately cowpea grains have shown lesser susceptibility to fungal infection and aflatoxins contamination compared to other staple crops such as maize and groundnut (Houssou et al., 2009). This encourages consumption of cowpea grains for mitigation of hunger and protein-energy deficiency. But cowpea is not consumed only in grain form. It is also processed into flour which is either used immediately or stored for further utilisation for the preparation of cakes

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(*Ata* and *adowe* in Benin, *Akara* in Nigeria, *Kose* in Ghana, etc.). The use of cowpea flour as a starting material for food processing is beneficial to food processors since it reduces the drudgery associated with initial grain processing operations and shortens the processing time. Concomitantly, the potential of the industrial production of ready-to-use cowpea flour in West Africa is high. In the currently set up factories, processed cowpea flour is packaged in polyethylene bag and could be stored as such for several months prior to sale.

Although, cowpea in whole grain form is resistant to aflatoxin contamination (Houssou et al., 2009), the susceptibility of processed flour to toxigenic fungi growth and subsequent toxin production has not been demonstrated. Cowpea processing into flour involves operations such as steeping and heat treatment/drying of the grains (Phillips et al., 2003). Recently we demonstrated that cowpea could loose its resistance following heat treatment at 121°C (Houssou et al., 2008). This indicates that at this temperature, it is possible that aflatoxin production inhibitory properties of cowpea grains can be lost during processing into flour. The extent of fungal growth and aflatoxin production as the result of this loss of inhibitory properties of cowpea needs to be investigated in order to establish the safety of the cowpea flour for long storage time periods. The main objective of the present work is to evaluate the influence of steeping and drying temperatures on the susceptibility of processed cowpea flours to fungal growth and aflatoxin production.

MATERIALS AND METHODS

Raw material and preparation of cowpea flour

Maroon seed coat variety, one of the most utilised cowpea variety for flour-based cowpea foods, was selected for this study. Initial sample of 05 kg of this cowpea variety was collected at the local market in Porto-Novo, Republic of Benin. The overall initial moisture content of this sample was 11.5%. This initial sample was divided into fifteen sub samples and used for different cowpea flours preparation. Duplicate cowpea flours were processed as described by Phillips et al. (2003), by steeping cowpea grains into the water for 3 min followed by drying at 35, 40, 50, 60, 80, 100 and 120°C to a moisture content of about 10% moisture content. Non steeped grains were also dried in the same conditions as 3 min steeping. Dried cowpea samples were manually dehulled using clean mortar and pestle. Dried and dehulled cowpea samples were milled into flours and sieved through a 450 µm screen. From a preliminary experiment, we found that increasing the steeping time from 3 to 6 min has no influence on the amount of aflatoxins produced in cowpea flour based media (data are not shown).

Formulation of cowpea flour based media

Cowpea flours were sterilized by electron beam irradiation at 30 kGy at room temperature and used for Cowpea Flour Agar solid media (CFA) preparation as described by Houssou et al. (2008). Five grams of sterilized cowpea flour were placed in 9 cm diameter Petri dish and autoclaved agar solution (20 g/L) added. The mixture was thoroughly mixed and left to solidify under a fume cupboard. Untreated (non steeped and non dried) sterilized cowpea flour based medium and YES - solid medium (20 g/L yeast extract [Merck Darmstadt, Germany], 150 g/L Sucrose and 20 g/L agar) were used as controls. YES is a good aflatoxin-production inducing medium.

Growth of fungi and aflatoxin production

The growth and aflatoxins production of *Aspergillus flavus* inoculated in solid Cowpea Flour Agar (CFA) media were determined for *in vitro* evaluation of the effects of processing parameters on the susceptibility of processed flours to fungi development and aflatoxin production.

Growth condition of fungi

One hundred micro litres (100 µl) of spore suspension of aflatoxin producing strains *(Aspergillus flavus*) previously isolated from naturally infected cowpea seeds (Houssou et al., 2008) were inoculated to CFA and incubated at 30°C for 7 days. After incubation period, the fungal colony diameter was measured and thereafter 3 to 5 agar plugs weighing 02 g collected from each plate. One plug was collected from the centre of the colony while the rest were picked from the periphery, wrapped in aluminium foil and immediately stored at - 20°C until analysed for aflatoxins.

Aflatoxin analysis

Extraction and clean-up procedure of samples

The aflatoxin extraction procedure was carried out as described by Pons (1979). The 2 g agar plugs collected were extracted with methanol, after which colour-pigments were removed by zinc acetate. Further clean up was performed by extraction with dichloromethane and column chromatography on cellulose and silica gel. Aflatoxins were eluted with dichloromethane-acetone (80:20, v/v) which was evaporated off and the residue quantitatively transferred into 10 ml of High Performance Liquid Chromatography (HPLC) grade dichloromethane. Five millilitres were evaporated to dryness and the final residue was kept in the freezer at - 20°C until HPLC analysis was carried out. Then the residue was dissolved in 0.1-1.0 ml of HPLC mobile phase (methanol -0.1 M sodium dihydrogen phosphate (77:23, v/v) adjusted to a pH of 3.35 with o-phosphoric acid) and used for HPLC.

Aflatoxin standard preparation and HPLC analyses

Aflatoxin standards were obtained from Sigma Chemical Co. (St Louis, MO, USA), Standard stock and HPLC working solutions were prepared by evaporating and dissolving in mobile phase consisting of methanol-acetonitrile-water (10:30:60, v/v/v) to give a concentration of 0.1 µg/ml for aflatoxins B_1 and G_1, and 0.03 µg/ml for B_2 and G_2.

Final residue of aflatoxins was re-dissolved in 200 µl of the HPLC mobile phase and analysed. The analysis was performed by reversed-phase liquid chromatography with post-column iodine derivatization. Separation of aflatoxins was carried out on a Spherisorb S5 ODS-1 column of dimension 25 × 4.6 mm packed with 5 µm particles (Phase Separations Inc, Norwalk, CT, USA)

Table 1. Fungal growth (expressed as diameter in cm) and mean aflatoxins B_1 and B_2 levels (µg/kg) in CFA inoculated and YES control plates.

Media	Steeping time (min)	Drying temperature (°C)	Fungal growth (cm)	Average AFB_1 and AFB_2 (µg/kg)	Total average aflatoxins (µg/kg)
	0	0	8.2	4.41 - 0.10	4.51[a]
	0	35	8.0	4.35- 0.05	4.40[a]
	0	40	8.1	7.00 - 0.07	7.07[ab]
	0	50	8.5	21.61 - 0.41	22.02[ab]
	3	35	8.4	16.42 - 0.14	16.44[ab]
	3	40	8.4	10.70 - 0.054	10.75[ab]
	3	50	9	21.01 – 0.09	21.10[ab]
Cowpea flour medium	0	60	> 9	43.40 - 1.24	44.64[c]
	0	80	> 9	55.62 - 1.62	57.24[c]
	0	100	> 9	72.18- 2.82	75.00[c]
	0	120	> 9	70.56 - 1.52	72.10[c]
	3	60	> 9	26.77 - 0.68	27.45[b]
	3	80	> 9	30.07- 0.67	30.74[b]
	3	100	> 9	34.73 - 1.05	35.78[b]
	3	120	> 9	30.91- 1.12	32.04
YES medium	> 9		98.11 - 0.18		98.23[d]

Values followed by different superscript letters are significantly ($p < 0.05$) different from each other.

maintained at 35°C. HPLC mobile phase flow-rate was 1.2 ml min^{-1} and post column iodine derivatization of aflatoxins B_1 and G_1 was achieved using saturated iodine solution according to the procedure of Shepherd and Gilbert (1984). Iodine was pumped at flow rate of 0.4 ml x min^{-1} using an Eldex precision metering pump (Eldex Laboratories Inc., San Carlos, CA, USA). Derivatization tube consisted of stainless steel tubing (5 m x 0.3 mm) maintained at 75°C. Excitation and emission wavelengths used were 360 and 440 nm, respectively. Aflatoxins were identified by their retention times and peak areas used to determine their concentration in the samples with reference to standard curves obtained by aflatoxin standard solutions under identical conditions. The detection limits were 0.04 µg/kg for B_1 and B_2 and 0.06 µg/kg for G_1 and G_2 respectively

Statistical analyses

Mean of fungal growth (cm) and mean aflatoxins (µg/kg) detected were calculated and compared using analysis of variance technique (ANOVA, SPSS 9.0 software). P-value < 0.05 was considered significant.

RESULTS

Aflatoxin concentrations detected in these CFA were compared with those recorded in YES medium used as positive control. After the incubation period, the *Aspergillus flavus* used was able to grow on all media, that is, YES and CFA including CFA from untreated sample. Table 1 shows fungal growth expressed as diameter in cm. The minimum diameter of colony measured was 8.0 cm on CFA. The development of the colonies was more pronounced in CFA made from the flour treated with higher temperatures (≥ 60°C) as compared to those treated with relatively low temperature (≤ 50°C). The intensity of the growth was more pronounced on YES medium as compared to that on CFA media (Plate 1).

The total concentrations of aflatoxins (B_1+B_2) in the CFA were directly proportional to drying temperature of the grain used for flour production (Table 1). There was a significant ($p<0.05$) positive correlation ($r = 0.64$) between growth of fungi and aflatoxin production. The concentration of aflatoxins detected in CFA prepared with flour processed using lower drying temperatures (35-50°C) ranged from 4.40 to 22.02 µg toxin /kg medium, while higher concentrations ranging from 27.45 to 75.00 µg/kg were detected in CFA prepared with flour produced using higher drying temperatures (60 -120°C). Low concentration (total average aflatoxins detected of 4.51 µg toxin /kg medium) was detected in CFA control prepared with untreated cowpea flour (non steeped and non dried grain) (Table 1). In YES medium known as good aflatoxins inducing medium and high aflatoxin (B_1+ B_2), a total average aflatoxin detected was 98.23 µg toxin/kg medium.

Steeping of cowpea also affected aflatoxin production in CFA media an effect which was dependent on drying temperatures (Table 1). When cowpea grains were dried at lower temperatures (35-50°C) no significant difference was observed at $P = 0.05$ between the overall means of aflatoxin concentration in CFA prepared from non steeped

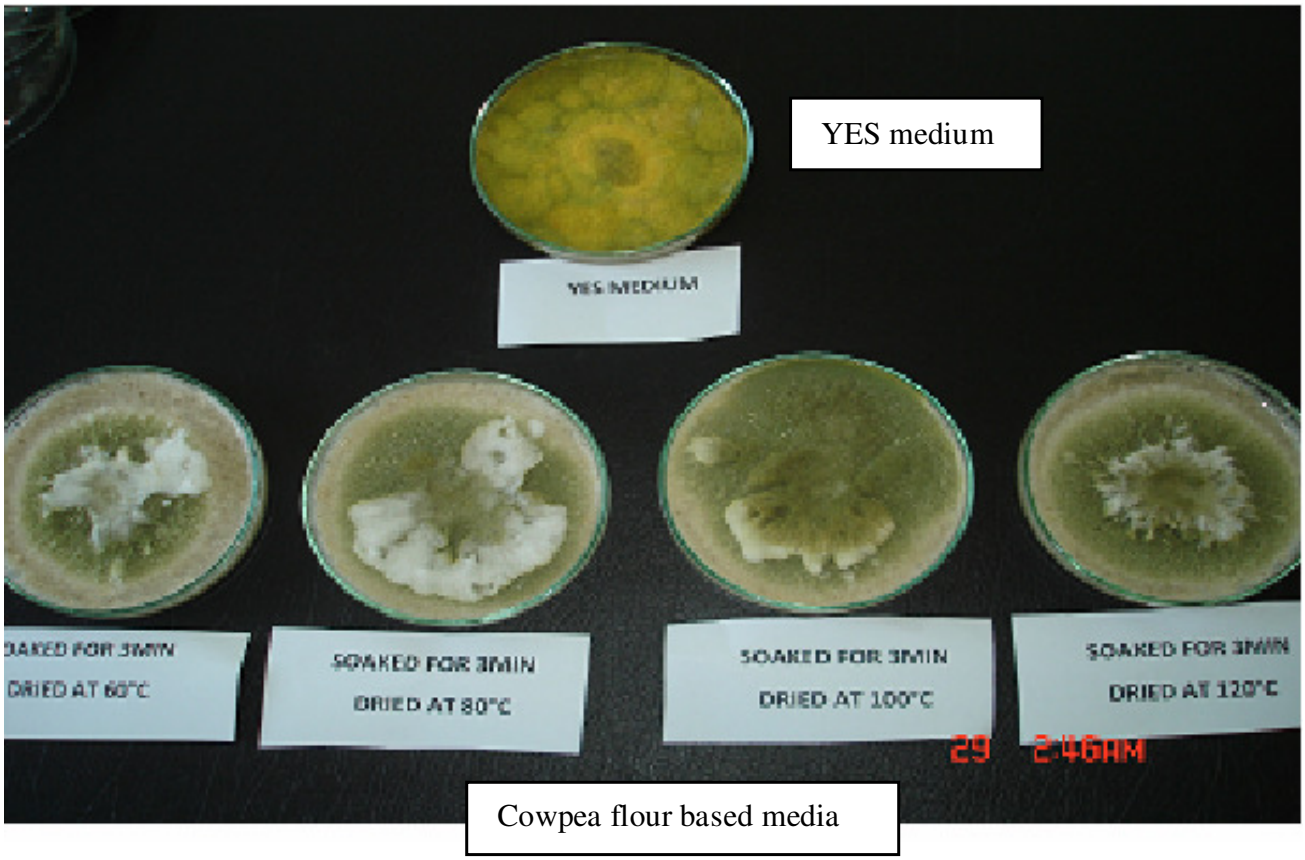

Plate 1. Growth intensity of the fungi on YES and cowpea based media.

steeped cowpea grains flour (11.16 ± 9.49 µg toxin /kg medium) and the concentration in CFA prepared from 3 min steeped grains flour (16.09 ± 5.18 µg toxin /kg medium).

However at higher drying temperatures of 60-120°C, the mean aflatoxin concentration in CFA prepared with non steeped grains flour was 62.24 ± 14.08 µg toxin/kg medium which was significantly ($P < 0.05$) higher than the mean concentration (31.51 ± 3.44 µg toxin /kg medium) in CFA formulated with steeped grains for 3 min.

DISCUSSION

This study has shown that steeping time and drying temperature of cowpea grains during processing into flour are important factors / variables that must be taken into consideration to preserve the quality of flour with respect to fungal infection and aflatoxin production. The enhanced aflatoxin production in the CFA following elevated drying temperature of grains as observed in this study may be attributed to the destruction of the substances with potential aflatoxin-formation inhibitory properties in cowpea grain as earlier demonstrated (Houssou et al., 2008; 2009). However, at higher temperatures (≥ 60°C) this effect is reduced when cowpea grains are steeped before drying. In fact, the average of aflatoxin detected was lower on steeped grain-formulated CFA than on non steeped grain flour formulated agar. We attribute this to a relatively slow drying of humidified grains compared to those non humidified where the drying is faster dragging a rapid destruction of potential aflatoxin production inhibitory substances in cowpea grains. Elsewhere, Sinha and Kawatra (2005) showed that steeping cowpea in water contributes to the reduction of its polyphenolic substances content such as tannin. In the same way, Phillips et al. (1983) demonstrated that humidifying cowpea grains before drying at 100°C slows down the destruction of trypsine inhibitors. Polyphenolic substances and trypsine inhibitors have been reported to have antifungal properties in cowpeas (Mahoney and Molyneux, 2004). Concomitantly, one would have expected more aflatoxin production on the CFA from steeped cowpea grains flour, which was not the case in the present study. Indeed our earlier work demonstrated that cowpea seed coat which has a higher

concentration in polyphenolic substances (Plahar et al. 2001), does not play any role in cowpea resistance to mycotoxin production (Houssou et al., 2008). Thus, other substances in cowpea with aflatoxin production inhibitory properties could be at play. These may include α and β-antifungal proteins, vicilins (7S storage proteins); phytic acid and enzyme inhibitors (Digrak et al., 1999; Ye et al., 2000; Melo et al., 2002; Rose et al., 2003; Ramakrishna et al., 2006).

Conclusion

It was concluded that treatment of cowpea grains to higher temperatures could render cowpea flour more susceptible to fungal infection and subsequent aflatoxin production. Reciprocally, treatment with lower temperatures could mitigate susceptibility of cowpea flour to aflatoxigenic fungal attack. Using higher drying temperature, it was observed relatively low aflatoxin production with media made with steeped cowpea grains for 3 min and dried at elevated temperatures varying between 60 and 120°C, compared with no steeped cowpea grain and dried to the same temperatures. Non-steeped or steeped cowpea grain in water for 3 min followed by drying at low temperature (≤ 50°C) seems to have no altered aflatoxin inhibitory activity in cowpea leading to minimal risk of fungal growth and aflatoxins contamination. Further studies on identification and characterization of these aflatoxin inhibitory substances are necessary.

ACKNOWLEDGEMENTS

This work was carried out under the project “Capability Building for Research and Quality Assurance in Traditional Food Processing in West Africa" and funded by the Danish International Development Assistance (DANIDA). The Toxicology Laboratory of the Food Research Institute (FRI) Accra, Ghana provided the technical and scientific support for aflatoxin analysis. We particularly thank George Anyebuno and Foster Mensah at FRI for their technical support.

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