Journal of Agriculture and Ecology, 2017, Vol.3, 41-51
http://saaer.org.in

Research Article **Open Access**

Peroxidase actvity, its isozymes and deterioration of pearl millet [*Pennisetum glaucum* (L.) R. BR.] flour during storage

P Goyal[a], MK Berwal[b], Praduman[c] & LK Chugh[a]

[a]CCS Haryana Agricultural University Hisar 125004, India,

[b]Division of Crop Improvement, ICAR-CIAH, Bikaner-334006, Rajasthan India

[c]ICAR-Indian Institute of Oilseeds Research, Government of India, Hyderabad 500030, India

Corresponding author: P Goyal, E-mail: goyalpreetigoyal@gmail.com

Copyright ©2017 Goyal et al., This is an open access article published under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Preferred citation for this article: Goyal P, Berwal MK, Praduman & Chugh LK. 2017. Peroxidase actvity, its isozymes and deterioration of pearl millet [*Pennisetum glaucum* (L.) R. BR.] flour during storage. *Journal of Agriculture and Ecology*, 3: 42-51; http://doi.org/10.53911/JAE.2017.3107.

Abstract

Development of off odour and flavour is an old and unresolved problem associated with pearl millet flour during storage and is the major hindrance for their consumer acceptability. In this study role of peroxidase, lipids and phenolics in deterioration of quality of pearl millet in control and stored flour of high (HHB 94) and low (ICMA 94222 x 78/71) rancid genotype was determined. Fat content, fat acidity, free fatty acids, total phenols, C-glycosylflavones content and peroxidase activity of HHB 94 were higher than that of ICMA 94222 x 78/71. Storage of flour for 8 days of these two genotypes showed significant increase in fat acidity and free fatty acids. Storage had no effect on C-glycosylflavones content whereas peroxidase activity reduced significantly with storage time. Isozyme profile showed that pearl millet grains had 3 isozymes of peroxidase and banding pattern was similar in both HHB 94 and ICMA 94222 x 78/71. While banding intensity represented that ICMA 94222 x 78/711 had lesser peroxidase activity as compared to HHB 94. The result suggested that peroxidase activity, fat acidity and free fatty acid value were usually correlated.

Key words: Pearl millet, peroxidase; isozymes, storage

Introduction

Poor shelf life of pearl millet flour because of development of off odour shortly after

grain milling is a major constrain to wider acceptability by the consumers and quantitatively high utilization in food industry. It has been reported that pearl millet has many nutritious and medical functions (Obilana & Manyasa 2002; Yang et al. 2012).The mineral profile of pearl millet is also better than other cereals. However, the availability of these minerals is low due to certain inherent factors. Although it is good, as far as nutritive value and product development is concerned, but major constrains that obstacle its diversified utilization is rapid development of off odour in its flour during storage. Formation of volatile compounds (Thiam et al. 1976), hydrolytic cleavage of fat (Kaced et al. 1984), changes in fatty acid composition of lipids (Lai & Varriano-Marston 1980), presence of phenolics in grains and their enzymatic degradation in flour (Reddy et al. 1986; Yadav, 2003; Sharma, 2006; Berwal et al. 2016 & 2017) have been identified as the contributing factor for generating rancid odour in pearl millet flour. Presence of high level of activity of peroxidase in pearl millet also causes development of rancid odour (Chavan & Hash 1998). Peroxidase, a member of a large group of enzymes called the oxidoreductases, is considered to have an empirical relationship to off-flavours and off-odours in foods. It is related to the "beany" and "greeny" flavor of soybean (Anil & Tilak 2004). Chavan and Hash (1998) in their studies identified high activity of peroxidase as the sole cause for generation of off odour. Banger et al. (1999) showed that water soluble phenolics and peroxidase activity concentrated mainly in germ fraction of grain appeared to be responsible for odour generation in stored pearl millet meal. Yadav (2003) reported that phenol content and activity of peroxidase might play an important role in generating off flavour during storage of flour. This contention is further supported by experiments conducted with defatted flour, dephenolic flour and flour with inactive peroxidase produced using blanching and other such treatments (Sharma, 2006). To determine relationship between peroxidase activity present in grains and magnitude of off odour developed in stored pearl millet flour knowledge of the kinetic properties of peroxidase of pearl millet grain is necessary. We have recently purified and characterized peroxidase from pearl millet grains (Goyal & Chugh 2013). Much of the difficulty in understanding peroxidase is due to the presence of multiple isozymes and as many as 73 isoperoxidases characterized in the model plant *Arabidopsis thaliana* (Welinder et al. 2002). Two cathodic peroxidases were purified from durum wheat flour (Iori et al. 1995). Electrophoretic studies of 21 pearl millet lines have revealed 5 different peroxidase isozymes (Manivannan et al. 2013). Converso and Fernandez (1995) found three cationic peroxidase isozymes in wheat germ.

Different factors that could be involved in causing rancid odour, however, have been studied independent of each other by the investigators named above. Thus, overall picture about the cause(s) of instability of pearl millet flour is not yet known clearly. Nevertheless, from the available information, it appears that phenolic compounds in high concentration and/or high

level of peroxidase activity in grains might be the major factors responsible for poor keeping quality of pearl millet flour. Hence, the present investigation was undertaken to ascertain role of fat content, phenol content and activity of peroxidase in development of rancidity in stored pearl millet flour. For conducting this experiment, two genotypes HHB 94 and ICMA 94222 x 78/71 were selected. These genotypes differed from each other in their chemical/biochemical composition as well as in development of off odour (Yadav 2003; Sharma 2006; Praduman 2006). Peroxidase isozymes were not studied in pearl millet grains yet. Now, Polyacrylamide gel electrophoresis was used to separate peroxidase isozymes from mature pearl millet grains. Moreover, contribution of different isozymes of peroxidase via their interaction with naturally occurring phenolic substrates like C–glycosylflavones towards development of off odour is yet to be explored.

Materials and Methods

Plant material

The present investigation was carried out on grains of two pearl millet hybrids viz. HHB 94 (F_2 generation) and ICMA 94222 x 78/711 (F_1 generation). The mature grains were procured from Bajra Section of the Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar.

Chemicals

All the chemicals and biochemicals used during the present course of investigation were purchased from either of the following: Sigma Aldrich Chemical Company (St. Louis, M.D., USA); Himedia Laboratories Limited, Bombay; Sisco Research Laboratories Pvt. Limited, Bombay; SD Fine-Chem. Ltd., Mumbai. All the chemicals were of the high purity analytical grade.

Preparation and storage of flour

Flour was prepared in a cyclotec grinding mill (Foss Analytical AB, Sweden) using 0.8 mm screen. Flour thus prepared was either used immediately for chemical analysis or for conducting experiments on stored flour. For investigating effect of storage, it was stored in glass beakers covered with aluminium foil at 37 ^{o}C in a BOD incubator at ambient humidity for specified period of time i.e. 4 or 8 days.

Biochemical analysis

The samples were analyzed for crude fat, fat acidity, free fatty acids, total phenols, C-glycosylflavone, off odour, and peroxidase activity before and after storage. Crude fat was determined using the standard method of AOAC (1990). The fat acidity and free fatty acids were expressed as potassium hydroxide required to neutralize the acids in a 100 g sample and

per g of fat respectively using phenolphthalein as an indicator (AOAC, 1990). Total phenols were estimated by the method of Malik and Singh (1980), using catechol as the standard. The method of Richert (1979) was used for the estimation of C-glycosylflavones. For rapid development of rancid odour and its evaluation method of Reddy et al. (1986) was used. All determinations were carried out in triplicate

Determination of peroxidase activity (EC 1.11.1.7)

Peroxidase was extracted and assayed by the method of Malik and Singh (1980). One unit of enzyme activity was defined as the amount of enzyme required to increase 0.1 unit absorbance min^{-1} under the test conditions.

$(NH_4)_2SO_4$ fractionation

To the crude extract obtained after centrifugation, solid $(NH4)_2SO4$ was added slowly with constant stirring so as to bring the saturation to 30%. After 4 h, the precipitate was removed by centrifugation (15,000 rpm, 4°C, 20 min), and $(NH4)_2SO_4$ was added to the supernatant to 65% saturation. The precipitates were collected by centrifugation as before and were dissolved in 5 mL of 0.1 M potassium phosphate buffer (pH 6.8). The resulting solution was dialyzed against the same buffer for 24 h. Every 4 h, the dialysis buffer was changed. The dialyzed $(NH_4)_2SO_4$ fraction was used for visualization of isozymes.

Visualization of isozymes

For visualization of isozymes, 30-65 % saturated $(NH_4)_2SO_4$ fraction of HHB 94 and ICMA 94222 x 78/711 containing 50 µg of protein was loaded in the wells of 7.5% polyacrylamide gel. Electrophoresis and staining was carried out by following the method of Davis (1964).

Staining

For staining of peroxidase isozymes, method of Guikema and Shermen (1980) was employed. The gels were stained in solution of 25% acetic acid containing 0.3% benzidine and 0.5% H_2O_2.

Protein estimation

Protein in crude and subsequent enzyme preparations at various stages of purification was quantitatively estimated by the method of Bradford (1976).

Results & Discussion

Crude Fat

Fat content of HHB 94 (7.4%) was higher than that of ICMA 94222 x 78/711 (5.7%) (Table1).

Fat acidity (FA)

Upon storage FA of fresh flour of both the hybrids increased with storage time. Comparatively rate of increase in FA in fresh flour of HHB 94 was higher than that of ICMA 94222 x 78/711(Table 1). For example, Fat acidity by 8th day of storage of flour of HHB 94 and ICMA 94222 x 78/711 was 474 and 240 mg KOH/100 g flour, respectively. Increase in FA of pearl millet flour stored for different time periods has been reported by many investigators (Chavan Kadlag et al. 1995; Palande et al. 1996; Chavan & Hash 1998; Bangar et al.1999; Yadav 2003; Praduman 2006; Sharma 2006; Nantanga et al. 2008; Jain 2013). Increase in fat acidity during storage is mainly due to action of lipase, causes bitterness and can make pearl millet meal unacceptable (Čepková et al. 2014).

Free Fatty acids (FFAs)

Measurement of free fatty acids provides a means of the relative rate of deterioration of flour. Upon storage, FFAs in flour of both the hybrids increased with storage time. Rate of increase in FFAs of normal flour of HHB 94 was higher than that of ICMA 94222 x 78/711 (Table 1) i.e. by 8th day of storage of flour of HHB 94 and ICMA 94222 x 78/711 concentration of FFAs increased by 6.4 and 4.8 fold, respectively. Similar effect of storage of untreated pearl millet flour on release of free fatty acids has been documented earlier (Patel & Parmeshwaran 1992; Sangwan 2005; Praduman 2006; Jain 2013).

Total phenols content

Content of total phenols of fresh flour of HHB 94 (232mg/100 g) was higher than that of ICMA 94222 x 78/711 (172 mg/100 g) Table 1. A range of 228-486 mg/100 g total polyphenols in pearl millet flour has been reported by Chavan and Hash (1998). Bangar et al. (1999) studied comparative distribution of phenolics in pearl millet grain fractions and reported that concentration of water soluble phenolics in the defatted meal (136 mg/100 g) was lower than in the germ (1216 mg/100 g). Variations in concentration of phenols reported in those studies might have been due to differences in the extraction solvents and method of extraction. During the present investigation, phenols were extracted in cold methanol. Further, there was no effect of storage on total phenols content of any type of flour of both the hybrids.

C-glycosylflavones

C-glycosylflavones content of normal flour of HHB 94 was higher than that of ICMA 94222 x 78/711. There was no effect of storage of all the types of flour on C-glycosylflavones content were observed (Table 1).

Table 1. Effect of storage on biochemical parameters responsible for deterioration of flour

Parameters	HHB 94	ICMA 94222 x 78/711
Crude Fat (%)	7.4±0.5	5.7±0.7
Fat acidity (mg KOH/100g flour)		
0 day	33.9±2	40±1
4 day	231.7±2.1	140±2.0
8 day	474.6±3.7	240±3.2
Free fatty acids (mg KOH/g fat)		
0 day	4.9±1	4.2±0.5
4 day	21.7±2.1	14±1.7
8 day	31.5±2.3	20.3±2.7
Peroxidase activity (units)		
0 day	320±5	260±5
4 day	240±1	140±1
8 day	194±2	110±1
Total phenols (mg/100g of flour)		
0 day	232±0.5	172±0.8
4 day	232±1	172±1
8 day	232±1	172±1.5
C-glycosylflavones content		
0 day	36.6±0.1	30.3±0.3
4 day	36.6±0	30.3±0.1
8 day	36.6±0.1	30.3±0.2

Units 0.1 change in OD/min is 1 unit of enzyme activity

Activity of peroxidase

Results on activity of peroxidase of HHB 94 and ICMA 94222 x 78/711 are presented in Table 1. Fresh flour of HHB 94 possessed 365 units of peroxidase activity which was higher than that present in flour of ICMA 94222 X 78/711 (281 units). Presence of peroxidase in pearl millet has also been observed by other investigators (Chavan & Hash 1998; Bangar et al. 1999). As reported earlier (Yadav 2003; Praduman 2006; Sharma 2006; Jain 2013), upon storage activity of peroxidase in control as well as modified (all the types) flour of both the hybrids was continuously decreased till 8 days of storage. Peroxidase is also important in

quality deterioration of buckwheat flour and flour of high peroxidase varieties tends to deteriorate quickly (Suzuki et al. 2010).

Development of off odour

Development of off odour in flour of these genotypes stored for 8 days was also recorded and presented in Table 2. It was observed that HHB 94 developed strong off odour (more than 4.0).

Table 2. Development of off odour during storage (8 day) of pearl millet flour

Genotypes	Rancidity flavour
HHB 94	5.0
ICMA 94222 x 78/711	2.7

Isozymes

Isozymes profile of peroxidase as depicted in Plate 1 HHB 94 and ICMA 94222 x 78/711 had 3 isozymes of peroxidase. In both the variety the pattern remained the same while the banding intensity corresponded to lesser peroxidase activity in ICMA 94222 x 78/711.

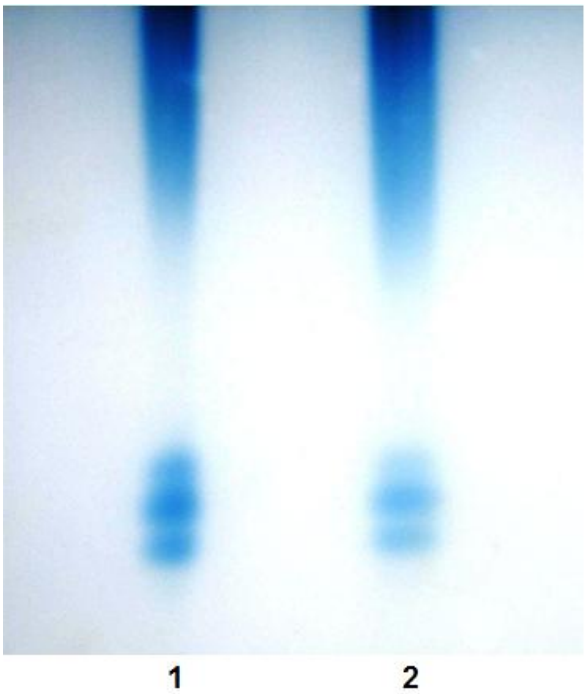

Plate 1. Isozymes profile of peroxidase obtained after $(NH_4)_2SO_4$ fractionation

Lane 1: HHB 94, Lane 2: ICMA 94222 x 78/711

Pearl millet grains of HHB 94 and ICMA 94222 x 78/711 exhibited three peroxidase isozymes with similar pattern (Plate 1) but the intensity of band indicated that ICMA 94222 x

78/711 grains had less peroxidase activity compared to HHB 94 and higher activity in HHB 94 was not due to any additional peroxidase isozymes. Manivannan et al. (2013) reported five peroxidase isozymes in pearl millet. Laugesen et al. (2007) found three distinct POXs in barley.

Further investigations are needed to identify, resolve and characterize isozymes in pearl millet. Experiments on identification and quantification of carbohydrates, association of the enzyme protein with phenolic compounds and interaction with C-glycosylflavones and/or other phenolics present in pearl millet, are needed before its clear cut role in mechanism of its involvement in the phenomenon of off odour development is identified.

Acknowledgements

Preeti thanks Department of Science & Technology, Govt. of India for providing financial assistance in the form of INSPIRE fellowship (SRF). The authors are also grateful to Chaudhary Charan Singh Haryana Agricultural University, Hisar for providing all the facilities required during the course of investigation.

References

Anil D & Tilak RM. 2004. Off-flavour development in soybeans: comparative role of some antioxidants and related enzymes. *Journal of the Science of Food and Agriculture*, 84:547–550.

AOAC 1990. Official Methods of Analysis. Association of Official Analytical Chemists, Washington, D.C.

Bangar MU, Bhite BR, Kachare DP & Chavan JK. 1999. Role of phenolics and polyphenol oxidizing enzymes in odour generation in pearl millet meal. *Journal of Food Science and Technology*, 36: 535-537.

Berwal MK, Chugh LK, Goyal P & Kumar R. 2016. Variability in total phenolic content of pearl millet genotypes: inbreds and designated B-lines. *Journal of Agriculture and Ecology*, 1: 41-49.

Berwal MK, Chugh, LK, Goyal P, Kumar R, and Dev Vart. 2017. Protein, Micronutrient, Antioxidant Potential and Phytate Content of Pearl Millet Hybrids and Composites Adopted for Cultivation by Farmers of Haryana, India. *International Journal of Current Microbiology and Applied Sciences*, 6(3): 376-386.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principal of protein dye binding. *Analytical Biochemistry*, 72: 248-254.

Čepková PH, Dvořáková Z, Janovská D & Viehmannová V. 2014. Rancidity development in

millet species stored in different storage conditions and evaluation of free fatty acids content in tested samples. *Journal of Food, Agriculture and Environment,* 12: 101-106.

Chavan JK & Hash CT. 1998. Biochemical constituents related to odour generation in some ICRISAT pearl millet materials. *ISMN*. 39: 151-152.

Converso DA & Fernandez ME. 1995. Peroxidase isozymes from wheat germ: purification and properties. *Phytochemistry*, 40: 1341-1345.

Davis BJ. 1964. Disc electrophoresis II. Method and application to human serum proteins. *Annals of the New York Academy of Scientices*, 121: 404-427.

Goyal P & Chugh LK. 2013. Partial purification and characterization of peroxidase from pearl millet [*Pennisetum glaucum* (L.) R. Br.] grains. *Journal of Food Biochemistry,* 38: 150–158.

Guikema JA & Shermen LA. 1980. Electrophoretic profiles of cyanobacterial membrane polypeptides showing heme dependent peroxidase activity. *Biochemica et Biophysica Acta*, 637: 189-201.

Iori R, Cavalieri B & Palmieri S. 1995. Cathodic peroxidases of durum wheat flour. *Cereal Chemistry*, 72(2): 176-181.

Jain S. 2013. Biochemical studies on shelf life of pearl millet [*Pennisetum glaucum* (L.) R. Br.] flour. *M.Sc. Thesis*, CCS HAU, Hisar, India

Kaced I, Hoseney RC & Varriano-Marston E. 1984. Factors affecting rancidity in ground pearl millet (*Pennisetum americanum* L. Leeke). *Cereal Chemistry*, 61(2): 187-192.

Kadlag RV, Chavan JK & Kachare DP. 1995. Effects of seed treatments and storage on the changes in lipids of pearl millet meal. *Plant Foods for Human Nutrition,* 47: 279-285.

Lai CC & Varriano-Marston E. 1980. Changes in pearl millet meal during storage. *Cereal Chemistry*, 57(4): 275-277.

Malik CP & Singh SB. 1980. In: *Plant enzymology and histoenzymology*. Kalyani Publishers, New Delhi, p. 53.

Manivannan A, Nimbal S. & Chhabra AK. 2013. Peroxidase isozyme characterization of elite genotypes of pearl millet [*Pennisetum glaucum* (L.) R. Br.]. *African Journal of Agricultural Research*, 8(28): 3662-3667.

Nantanga KMK, Seetharaman K, Kock LH & Taylor RNJ. 2008. Thermal treatments to partially pre-cook and improve the shelf life of whole pearl millet flour. *Journal of the Science of Food and Agriculture*, 88: 1892-1899.

Obilana AB & Manyasa E. 2002. Millets. In: P. S. Belton and J. R. N. Taylor (Eds.). pp. 177–217. Pseudocereals and less common cereals: Grain properties and utilization potential. Springer-Verlag: New York.

Palande KB, Kadlag RV, Kachare DP & Chavan JK. 1996. Effects of blanching of pearl millet seeds on nutritional composition and shelf life of its meal. *Journal of Food Science and Technology*, 32(2): 153-155.

Patel KV & Parmeshwaran M. 1992. Effects of heat treatment on lipid degradation in bajra flour during storage. *Journal of Food Science and Technology*, 29(1): 51-52.

Praduman. 2006. Grain quality of grey and yellow pearl millet (*Pennisetum glaucum* L. R. Br.). M.Sc. Thesis, CCS HAU, Hisar, India.

Reddy VP, Faubion JM & Hoseney RC. 1986. Odor generation in ground, stored pearl millet. *Cereal Chemistry* 63(5): 403-406.

Reichert RD. 1979. The pH – sensitive pigments in pearl millet. *Cereal Chemistry,* 56: 291-294.

Sangwan A. 2005. Nutritional evaluation and product development from white and yellow pearl millet varieties. M.Sc. Thesis, CCS HAU, Hisar, India.

Sharma A. 2006. Studies on the development of rancidity in stored flour of pearl millet (*Pennisetum glaucum* (L.) R. Br.). *M.Sc. Thesis*, CCS HAU, Hisar, India.

Suzuki T, Kim SJ, Mukasa Y, Morishita T, Noda T, Takigawa S, Hashimoto N, Yamauchi H & Matsuura-Endo C. 2010. Effects of lipase, lipoxygenase, peroxidase and free fatty acids on volatile compound found in boiled buckwheat noodles. *Journal of the Science of Food and Agriculture*, 90(7):1232-1237.

Thiam DA, Drapron R & Richrd-Holard D. 1976. Cause de dalteration des garines de millet de crop. *Annales de technologie agricole*, 25:253.

Welinder KG, Justsen AF, Kjaersgard IVH, Jensen RB, Rasmussen SK, Jespersen HM & Durox L. 2002. Structural diversity and transcription of class III peroxidase from Arabidopsis thaliana. *European Journal of Biochemistry*, 267: 6063-6081.

Yadav RK. 2003. Biochemical changes during storage of pearl millet (*Pennisetum glaucum* (L.) R. Br.). *M.Sc. Thesis*, CCS HAU, Hisar, India.

Yang X, Wan Z, Perry L, Lu H, Wang Q, Hao C, Li J, Xie F, Yu J, Cui T, Wang T, Li M Ge QH. 2012. Early millet use in northern China. *Proceedings of the National Academy of Sciences,USA, pp. 1-5.*