J Biochem Tech (2012) 3(5):144-146
ISSN: 0974-2328

Reduction of phytate content in unfermented whole grain wheat flour dough using permeabilized phytase active *Candida versatilis* mutants

Sadanandan B*, Varadaraj M C, Ramesh Govindarajan T, Channarayappa, Lokesh K N

Received: 27 August 2012 / Received in revised form: 14 October 2012, Accepted: 08 December 2012, Published online: 29 December 2012

Abstract

High phytic acid (PA) content in whole wheat flour products reduces the bioavailability of nutrients, especially dietary minerals. Monogastric animals cannot breakdown PA, an organophosphorus compound as they do not produce the enzyme phytase. Hence phytate gets excreted from the system leading to phosphorus deficiency and accumulates in the environment. Accumulation of PA also causes serious soil and water pollution during animal husbandry. In non-fermented food products the benefit of activation of innate grain phytases or microbial phytases cannot be exploited. In the present study the use of freeze-thaw permeabilized phytase active mutant yeast cells to reduce PA content in unfermented foods has been successfully tested. Candida versatilis mutants, UVY 505 and EMY 505 used in this process were able to bring about phytate reduction of 24.32 to 45%. The substantial but not complete removal of PA here also helps to derive the cardiovascular and other health benefits of PA acknowledged of late. The method developed is simple, rapid, chemical free, nontoxic, economical and ideal for food applications.

Keywords: Phytic acid, Phytate, Phytase, Whole wheat flour, Candida versatilis, Permeabilization

Sadanandan B

Department of Food Microbiology, CSIR-Central Food Technological Research Institute (CFTRI), Mysore 570020
*Email: bindu.sadanandan@gmail.com

Varadaraj M C

Department of Food Microbiology, CSIR-Central Food Technological Research Institute (CFTRI), Mysore 570020

Ramesh Govindarajan T

Molecular Toxicology Laboratory, Center for Biotechnology & Biomedical Sciences, Department of Biology,
Norfolk State University, Norfolk, VA 23504, USA

Channarayappa, Lokesh K N

Department of Biotechnology, M S Ramaiah Institute of Technology, Bangalore-560054

Introduction

Unleavened flat breads prepared from whole wheat flour are consumed by people in most parts of the world. Whole wheat flour used in dough preparation is not refined and contains bran rich in phytic acid. Phytic acid is considered as an antinutritional factor as it chelates protein, carbohydrate and divalent cations (especially Mg+2, Ca+2, Zn+2, Fe+2, Fe+3), thereby making them unavailable for absorption (Pallauf and Rimbach1996; Martin et al. 2006; Cao et al. 2007; Liu et al. 2008 , Lopez et al. 2002). Whole wheat leavened bread contains 4.3-6.8 mg/g dry matter of PA whereas unleavened wheat bread contains 9.2-19.5 mg/g dry matter of PA. On an average, the daily intake of phytate has been estimated to be 2000-2600mg for vegetarian diets especially in rural areas of developing countries and 300-1300 mg for mixed diet (Greiner and Konietzny 2006). As minerals, vitamins and dietary fibre are bound together with phytate, a mechanical removal of phytate by removing the outer grain layers will lower the nutritive as well as the organoleptic qualities of the resultant product. However to retain these qualities, the phytate needs to be removed enzymatically. Phytase is the enzyme that hydrolyzes phytate. It is naturally present along with phytate in wheat grains, but usually gets inactivated due to long term storage of wheat as well as during processing of wheat into its products (Lopez et al. 1998). Dephosphorylation of phytic acid to even a minimum extent can have a significant effect on mineral absorption as evident from the suckling rat pup model demonstrated by Lonnerdal et al. (1999).

Fermentation is a good option for activation of innate grain phytases but is not desirable in non-fermented food products as it would alter the textural and organoleptic properties of the finished product. Permeabilization of cells for better access to cell associated phytase is a good option (Bindu et al 1998; Kaur and Satyanarayana 2010). In the present study we have used phytase active permeabilized cells of the EMS & UV mutants of Candida versatilis CFR 505 to effect reduction in whole wheat flour dough PA content. As the phytase produced here is cell associated freeze-thaw using ice-salt mixture and liquid nitrogen have been used to access the enzyme in situ. Of the two methods tried liquid nitrogen permeabilization was found to be more effective. This process is ideal for high phytate traditional unfermented foods as phytate reduction is achieved without any physical or organoleptic changes as evidenced from sensory evaluation of the flat bread made out of the dough. The yeast used in this process is food grade with GRAS status hence poses no health hazards. The permeabilization method chosen is also a physical one

free of chemicals. The use of liquid nitrogen permeabilized phytase active food yeasts is a simple, rapid, nontoxic and economical method to effectively reduce phytate in whole grain unfermented foods.

Materials and Methods

Cultures

Local isolate (CFTRI, Mysore, India) from fermented rice and gram mix Candida versatilis CFR 505 as the control along with mutants obtained via Ethyl Methane Sulfonate treatment & Ultra Violet irradiation namely EMY 505 and UVY 505 have been used for the study.

Phytase screening agar method for qualitative detection of Phytase production

The wild type and mutants of C versatilis CFR 505 were screened for phytase production through point inoculations of the individual cultures on phytase screening agar medium according to the method described by Howson and Davis (1983). After 36h of incubation at 30^0C, the ratio of colony diameter to the distance of agar clearing from the colony edge was measured and used to detect extracellular/cell free phytase production.

Isolation of Cell associated Phytase

Results of the above experiment served as positive control for this assay. Cell associated phytase activity was determined using cells obtained from 20h old culture broth. Aliquots of cells in 2g each were then homogenized in a pre-chilled pestle and mortar using glass beads of 212-300µm (Sigma, St. Louis, Mo., USA) with protease inhibitor cocktail of 2mmoll-1 PMSF, EDTA and β-mercaptoethanol in 10% glycerol. The homogenate was centrifuged at 12000g for 15 min at 4^0C and the resultant cell free extract contained the cell associated phytase and was used for the assay.

Assay of Cell associated Phytase activity

Phytase activity was determined by measuring the liberated inorganic phosphate from phytate according to the method described by Ullah and Gibson (1987). The assay was performed at a pH level of 5.0 for 35min at 50^0C in a thermostatically controlled water bath. One unit (U) of phytase activity is defined as 1 µmole of inorganic phosphorus produced per min per ml at pH 5.0 and 50^0C.

Preparation of inoculum of wild type and mutants for permeabilization

Aliquots of sterile 250ml of potato dextrose broth taken in 1000ml Erlenmeyer flasks were inoculated with a loopful of the wild type and mutants individually and incubated for 18h at 30^0C in an orbital shaker at 200 rpm. Cells were harvested from the incubated culture broth by centrifugation at 1100g for 10 min at 4^0C. The resultant supernatant was discarded and the cell pellet washed twice with 0.1M sodium acetate buffer at pH 5.0. The cells so obtained were subjected to permeabilization by freezing and thawing.

Ice-salt Freeze-thaw method of permeabilization

The wet cells of the wild type and both the mutants, individually in aliquots of 1g quantity were suspended in 10ml of sodium acetate buffer. This suspension was frozen in ice-salt freezing mixture for about 10 minutes and then thawed at 26^0C again for 40 seconds under tap water. This process of freezing and thawing was repeated for up to 20 cycles. The resultant permeabilized cells were washed with sodium acetate buffer and used for assay of phytase activity.

Liquid Nitrogen Freeze-thaw method of permeabilization

Freezing of the wet cells of the wild type and two mutants, individually was carried out in liquid nitrogen for 20 seconds, followed by thawing at 26^0C for 40 seconds under running tap water. The process was repeated for about 4 cycles. The resultant permeabilized cells were washed with sodium acetate buffer and used for assay of phytase activity.

Process for preparation of dough for reduction of phytic acid

Aliquots of whole wheat flour in 100g quantities each were taken in sterile stainless steel vessels to which were added 2 x108 CFU of freeze-thaw permeabilized cells of the wild type C versatilis CFR 505 and the EMS and UV mutants (EMY 505 and UVY 505) individually. The added cells were mixed uniformly with the whole wheat flour using a sterile flat spoon. To the individual mixture of wheat flour respective and permeabilized yeast cells were added 45 ml of sterile distilled water, 1g table salt and kneaded well into dough with hand wearing sterile disposable gloves. Each of the prepared dough samples were divided into two equal parts, with one portion being kept at 26^0C for 30 minutes and the other portion for 24h at 10^0C. Phytic acid content was determined in 1g quantity of all the above mentioned samples of dough. The Unfermented whole wheat flour dough without C versatilis CFR 505 addition was used as a control for the substrate and as the organism control, non- permeabilized and permeabilized wild type cells of C versatilis CFR 505 were also used in the unfermented whole wheat flour dough.

Estimation of phytic acid

The method of extraction, purification and spectrophotometric determination of phytic acid was performed according to the method of Fruhbeck et al. (1995).

Results and Discussions

Phytase screening agar method for qualitative detection of Phytase production

Point inoculations of wild type and mutants in phytase screening medium failed to show any clearance of calcium phytate around the colonies indicating absence of extracellular phytase activity.

Isolation and assay of Cell associated Phytase

Cell associated Phytase was isolated and the Phytase activity was found to be 167.87 and 142.52 Ug-1cells for EMY 505 and UVY 505 respectively. The increase was 1.76 and 1.63 – fold respectively. In the permeabilized cells, there was an increase of 35 Ug-1cells, relative to the non-permeabilized cells. The ice-salt permeabilized cells exhibited an increased activity of 112 and 99 Ug-1cells for EMY 505 and UVY 505 respectively as against the permeabilized cells of the wild type. Liquid nitrogen permeabilized wild type cells, exhibited an increased phytase activity of 90 Ug-1cells in 4 cycles. The permeabilized cells of EMY 505 and UVY 505 showed an increase 197 and 170 Ug-1cells, respectively relative to the wild type.

Estimation of phytic acid

There was no reduction in phytic acid during storage of dough (control) as well as in dough added with non-permeabilized and permeabilized cells of wild type. In dough added with non-permeabilized cells of mutants too no reduction in phytic acid was observed. In a storage period of 30 minutes at 26^0C, the permeabilized mutant UVY 505 cells derived by freeze-thaw using ice-salt mixture and liquid nitrogen were able to bring about an 11.6 and 20.0% reduction in phytic acid level respectively. The reduction was 16.3 and 24.32% in 24h at 10^0C. For the permeabilized mutant EMY 505 cells however in a storage period of 30 minutes at 26^0C, freeze-thaw using ice-salt mixture and liquid nitrogen were able to bring about an 18.9 and 39.9% reduction in phytic acid level respectively. The reduction was 24.2 and 45.1% in 24h at 10^0C (Fig. 1). Hence as observed the reduction achieved with freeze – thaw permeabilized cells of mutant of EMY 505 was found to be higher than UVY 505 mutant

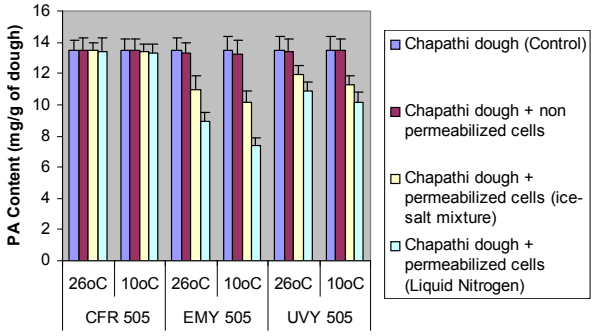

Figure 1: Effect of freeze thaw permeabilized cells of Wild type Candida versatilis CFR 505 and its mutants EMY 505 and UVY 505 on Phytic acid levels of whole wheat flour unleavened (Chapathi) dough. The results are shown as a representative of three independent experiments.

Conclusion

Consumption of whole grain flour (WGF) products has increased over the years due to the associated health benefits but bran present in WGF also contains PA which is an antinutritional factor. Hydrolysis of PA using phytase is seen as a way to reduce the risk of running into mineral deficiency in vulnerable groups such as child-bearing women, strictly vegetarians and inhabitants of developing countries (Greiner and Konietzny 2006). A biotechnological approach to reduce antinutritional factors like phytic acid in traditional non fermented whole wheat flour dough was attempted. Potent mutants of yeast culture isolated from food with cell associated phytase activity has been used. Simple methods of mutation and permeabilization have been used to achieve higher phytase activity. In dough added with freeze – thaw permeabilized cells of the mutants especially EMY 505 a significant reduction in phytic acid level was seen. Hence we have found that freeze thaw permeabilization of mutants of C versatilis using liquid nitrogen is a simple and rapid method to improve the accessibility and applicability of cell associated phytase activity. These phytase active cells can be effectively used to reduce phytic acid levels in unfermented whole wheat flour dough used to make flat breads and an American patent has been filed about the same (Sadanandan B and Chakravarathy 2005).

Acknowledgements

Bindu Sadanandan acknowledges University Grants Commission for Junior Research Fellowship and Council of Scientific Industrial Research for Senior Research Fellowship. The authors also acknowledge the encouragement & support provided by the Director, CSIR-Central Food Technological Research Institute, the Management & Principal of M S Ramaiah Institute of Technology.

Reference

Bindu S, Somashekar D, Joseph R (1998) A comparative study on permeabilization treatments for in situ determination of phytase of Rhodotorula gracilis. Lett Appl Microbiol 27:336–340

Cao L, W Wang, C Yang et al (2007) Application of Microbial Phytase in Fish Feed. J Enzyme and Microbial Technol 40:497-507

Fruhbeck G, Alonso R, Marzo F et al (1995) A modified method for the indirect quantitative analysis of phytate in foodstuffs. Anal Biochem 225:206-212

Greiner R, Konietzny U (2006) Phytase for food application. Food Technol Biotechnol 44:125–140

Howson S J and Davis R P (1983) Production of phytate hydrolyzing enzyme by some fungi. Enzyme Microb Technol 5:377-382

Kaur P, Satyanarayana T (2010) Improvement in cell-bound phytase activity of Pichia anomala by permeabilization and applicability of permeabilized cells in soymilk dephytinization. J Appl Microbiol 108:2041- 2049

Liu Z, Wang H, Xiu E et al (2008) Effect of Wheat Perling on flour phytase activity, phytic acid, iron and zinc content. LWT - Food Sci. Technol 41:521-527

Lonnerdal B, Jayawickrama L and Lien E L (1999) Effect of reducing the phytate content and of partially hydrolyzing the protein in soy formula on zinc and copper absorption and status in infant rhesus monkeys and rat pups. Am J Clin Nutr 69:490-496

Lopez HW, Coudray C, Bellanger J et al (1998) Intestinal fermentation lessens the inhibitory effects of phytic acid on mineral utilization in rats. J Nutr 128: 1192-1198

Lopez HW, Leenhardt F, Coudray C, Remesy C (2002) Minerals and phytic acid interactions: is it a real problem for human nutrition? Intl J of Food Sci & Technol37:727–739

Marti JA, Murphy RA and Power RFF (2006) Purification and physico-chemical characterisation of genetically modifed phytases expressed in Aspergillus awamori. Bioresource Technol 97:1703- 1708

Pallauf J and Rimbach G (1996) Nutritional Significance of Phytic Acid and Phytase. Arch Anim Nutr, 50:301-319

Sadanandan B and Chakravarathy VM (2005) Process for the preparation of Chapathi dough with reduced phytic acid level. United States patent application: 2005/0048165A1, Publication date: March 3, 2005

Ullah AHJ and Gibson DM (1987) Extracellular phytase (EC. 3.1.3.8) from Aspergillus ficuum NRRL 3135: purification and characterization. Prep Biochem17:63-91