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Study on Protease from Barley Tempeh and *in vitro* Protein Digestibility

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Abstract

In the present study, koji fermentation of barley seeds with *Rhizopous oligosporous* G-10 strain has a significant effect on the proteolytic activity and in vitro protein digestibility. The various process parameters were evaluated for the 100gm barley grains tempeh production, which was soaked for 8 hour in 500ml distilled water followed by boiling for 15 min in the presence of 1% NaCl and 1%glacial acetic acid and drying at 100 ^{o}C for 5 min. After pretreatment, barley grains were inoculated with 1% (v/w) spore suspension containing 10^{7} /mL and incubated at 30 ^{o}C for 36 hours. The maximum protease units (99.52 + 1.12 IU/g) were observed in fermented barley. The protein digestibility of 62% and 28% was found in fermented and unfermented barley during in vitro study.
Keywords: Koji fermentation, proteolytic, Rhizopous sp, protein digestibility.

1. Introduction

A fermented food from plant or animal origin are a part of the diet of the people in all over the world and is now oriented to develop low cost protein foods of plant origin to fulfill the protein (Danish *et al*., 2009; Mohammad *et al*., 2007). The second most abundant cereal grain in world is barley, consisting of 49-66% starch, 14-28 % dietary fiber and 9-22% crude protein, varies for different varieties (Kuswanto K and Rahayu, 2004). The preparation of many indigenous or traditional fermented foods remains today a household art for the improvement of nutritional value of the food through fermentation (Oscarsson *et al*., 1996). Microbial fermentation is considered as one of the oldest and most economical methods for food production and preservation. The fermentation process of cereals and legumes may increase the digestibility and bioavailability of proteins, carbohydrates, lipids, minerals and enhance the nutritional value such as vitamin content; shorten the cooking time and increase the microbial safety (McKeown N M, 2004). Therefore consumption of fermented food would lower food costs and promote better health (Behall *et al*., 2004a). Barely tempeh has been fermented through a process similar to soybean tempeh with Rhizopus spp, which is highly active proteolytic and lipolytic enzymes was suitable for producing tempeh from cereal grains (wheat, barley, oat, rye and rice) (Granfeldt *et al*., 1994). This early attempt to produce barley tempeh used dehulled andcracked barley (Hesseltine *et al*., 1967). However, commercial barley tempeh has not yet been produced. Previously, a patented barley tempeh procedure has been developed by fermentating whole pearled barley kernels (Gourmet korn) with selected strains of *R. oligosporus* (Berg *et al*., 2001). The fermentation process has recently been modified and applied on a new barley genotype (Karmose) with a high amyloseand β -glucan content. The modified process has been found to strongly reduce the phytate content while preserving minerals (Eklund-Jonsson *et al*., 2006) and also lower the glycemic index of barley tempeh.

The aim of the study is to prepare the barley tempeh through Koji fermentation by *Rhizopus oligosporous* G-10. The protease and in vitro protein digestibility of unfermented and fermented barley were study.

2. Material and Method

2.1. Culture

The *Rhizopus oligosporous* G-10 was procured from Microbiology Lab, Food and Biotechnology Research Centre, PCSIR Labs Complex Lahore. The strain was grown on PDA agar slant (Oxoid) for 48 h at 30 ^{o}C. The culture was then preserved at 4 ^{o}C and further shifting on the PDA slant at the interval of 30 days to keep them viable. The pH 5 of the medium was adjusted with 1N HCl / NaOH before sterilization at 121 ^{o}C for 15 min.

2.2. Inoculum preparation

Inoculum was prepared by transferring a 10 ml of sterilized distilled water in 48 hour old slant of *Rhizopus oligosporous* G-10. The spores were taken under the sterile

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conditions and inoculum used in this study has a concentration of 10^7 spores / ml.

2.3. Substrates

The substrate (Barely) which is used in this work was obtained from the Local Market (Lahore, Pakistan).

2.4. Solid-state fermentation

The Berg method (2001) for the barely tempeh was used with slightly modification. Hundred grams of dry barely seeds were soaked for 8 hour in the 1L glass beaker which was containing the 500 ml of distilled water. Dehulled the soaked barely by manually and then boiled for 15 minute by adding the 1% glacial acetic acid and 1% sodium chloride to avoid the contamination. The boil water drained off and barley was kept in oven at 80 ^oC for 5 minutes to get the required moisture level for the growth of the strain. Then prepared spore suspension of 1% was used to inoculate the room temperature boiled barley and packed in pre- holed 12 x 12 cm^2 polythene bags for barely cake formation at 30 ^oC for 36 hour . All the experiments were conducted in triplicate.

2.5. Process parameters

The various parameters were studied in koji fermentation of barley by *Rhizopus oligosporous* G-10.The various soaking time of barley in distilled water (4, 6, 8, 10, 12, and 14 hours), boiling time (5, 10, 15, 20, 25 and 30 minute), inoculum size (0.5, 0.75, 1, 1.25, 1.5 and 2 %), incubation temperature (25, 30, 35 and 40 ^0C) and different time interval of koji fermentation (24, 36, 48 and 72 h).

2.6. Crude extracts

Triplicate fermented barely (10 g) were mixed with 100 ml of 0.05 M phosphate buffer (pH 5) and homogenized in a blender machine (Eyela,Japan). The homogenized mixture were kept at room temperature for 30 min with frequent stirring, and then centrifuged at 10000 rpm for 10 min. The supernatant was used as crude enzyme extract.

2.7. Protease activity

Protease activity was determined by the method of Yang and Huang (Yang SS and Huang CI, 1994). The reaction mixture containing 2 mL of 1 % casein solution in 0.05 M phosphate buffer (pH 5) and 1 mL of enzyme solution were incubated at 60 ^oC for 15 min and the reaction was then stopped with the addition of 3 mL of 10 % trichloroacetic acid. After 10 min the entire mixture was centrifuged at 10000 rpm for 10 min at 4 ^oC and absorbance of the liberated tyrosine was measured with respect to the blank at 280 nm. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 μg of tyrosine per min under assay conditions.

2.8. Protein Digestibility

The *in vitro* protein digestion (IVPD) of barley flour was determined by using the Borchers (Ekpenyong TE and Borchers RL, 1979). A multienzyme technique was used for studied protein digestibility. About 2.60gm of barley flour was dissolved in 50ml of distilled water to give the amount of sample to contain 6.25 mg protein/ml and kept at 5^oC for 1hr. The pH of the sample suspension was adjusted to 8 with 0.1 N HCl / 0.1 N NaOH. Then 5ml of multienzyme mixture (1.6 mg /ml trypsin, 3.1mg /ml chemotropism and 1.3 mg /ml peptidase) were added to the sample suspension with constant stirring at 37 ^oC for 10 min. After incubation the pH of the sample suspension was measured and protein digestibility was calculated by formula IVPD=210.464-18.10X, where X is pH after 10 min digestion.

3. Result and Discussion

In the present study deals with soaking of raw barley in distilled water before Koji fermentation to minimize the hardness effect of barley to improve the growth of *Rhizopus oligosporous* G-10. The results were indicated that the maximum units (78.15 IU/gm) of protease were found after the 8 hour soaking of barley (Fig. 1). The researchers were reported that soaking of the cowpea reduced the antinutritional factors such as protease inhibitors, tannins, phytic acid and flatus-producing oligosaccharides (Ibrahim *et al*., 2002). The soaking increased lysine availability by 21% and 22% for maize and maize-cowpea mixtures in fermentation process was reported by (Nche *et al*., 1995). The soaking in distilled water and $NaHCO_3$ solution significantly reduced the contents of total free phenolics (85–88%) compared to raw seeds (Vijayakumari *et al*., 1995). In this study, soaking has considerable effect on the growth of the strain in barley tempeh.

The cooking has a significant effect on the barley koji fermentation by *Rhizopus oligosporous* G-10. The maximum protease units (80.15 IU /gm) were observed at 15 minutes boiling (Fig. 2). The African yambean was soaked in water for 24 hour and 30 min boiled and reduced the hardness (Marshall *et al*., 2006). The researcher reported that cooking of soaked cowpea further improved lysine availability by 68% and 31% for maize and maize-cowpea mixtures (Nche *et al*., 1995). Cooking has decreased the levels of lectin and trypsin inhibitor in fermented bean (Ana *et al*., 1998). In fermentation of cooked soybeans, proteases, lipases and phytases were produced and due to enzymatic degradation of macromolecules into of lower molecular weight and moist heat have destroying protease inhibitors and expose up the protein structure (Steinkraus *et al*., 1983; Nout and Rombouts , 1990).

The optimum level of inoculum is crucial in fermentation process; low density gives insufficient biomass and high density produces too much biomass and resulting depletion of the nutrients. The results indicated that 1% inoculum gave maximum units of protease (88.10 U/gm) (Fig.3). The spores suspension (1×10^6 spores/ml) was used as inoculum for tempeh from hardened chickpeas with *Rhizopus stolonifer* (Reyes-Moreno *et al*., 2000).

In the present work, the temperature has a significant effect on the growth of *Rhizopus oligosporous* G-10 in koji fermentation of barley for dense mycelia growth. A maximum unit (98.52 U/gm) of protease at 30^oC (Fig.4) is noticed. The optimum temperature of 35.8 ^oC was required for chickpea tempeh formation with *Rhizopus stolonifer* (Reyes-Moreno *et al*., 2000). It was reported that 30^oC was optimum temperature for douchi formation with mould fungus (Chuanlai *et al*., 2005). The inoculation of beans with *Rhizopus oligosporus* at various temperatures

followed by incubation at 30 °C resulted in both increased and decreased periods for the lag phase of fungal growth (Reu *et al*., 1995).

The time optimization in Koji fermentation is important for maximum mycelia growth and to avoid the stage of the sporulation in tempeh of barley with *Rhizopus oligosporous* G-10. The results of the study indicated that the maximum unit (99.52 U/gm) of protease was found at 36 hour of incubation time (Fig.5). The optimum time of 42.7 hour at 35.8 °C was reported for the chick pea tempeh with *Rhizopous stolonifer* by the researchers (Reyes-Moreno *et al*., 2000).

The results of the Omafuvbe *et al*, (2004) study indicated that the relative proteolytic activity in the fermenting soybean increased at 48 h of fermentation at 30- 40 °C. The workers reported that in fermented rice increased in protein level was observed at 24 h of the fermentation (Nnam and Obiakor, 2003). The genus *Rhizopus* secreted enzymes like protease in the tempeh from the substrates like soyabean, wheat, sorghum (milo), oats, rye, barley and corn which improved the quality of the tempeh (Kathleen *et al*., 1993; Waraw Krausong and Yoshikitani, 2005).

The *in vitro* protein digestibility of fermented and unfermented barley was found 62.56% and 28.55% (Table.1). Fermentation was found to create a significant effect on *in vitro* protein digestibility of barley tempeh. The proteolytic enzyme increased the protein digestibility, which was secreted in fermentation. Similar findings show that microorganisms were found to produce proteolytic enzymes during fermentation, which dissociate and degrade proteins, rendering them more accessible to proteases, and hence increased digestibility (Yagoub, 2003; Khaterpaul and Chauhan, 1991; Aderibigbe and Schink ,1990).

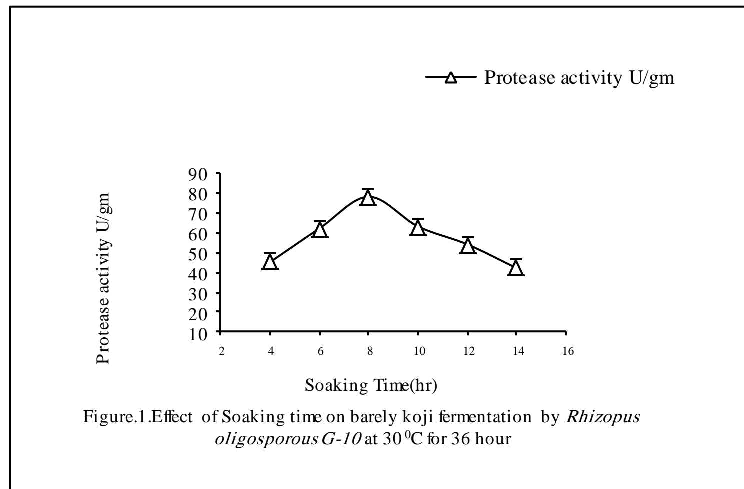

Figure.1.Effect of Soaking time on barely koji fermentation by *Rhizopus oligosporous G-10* at 30 °C for 36 hour

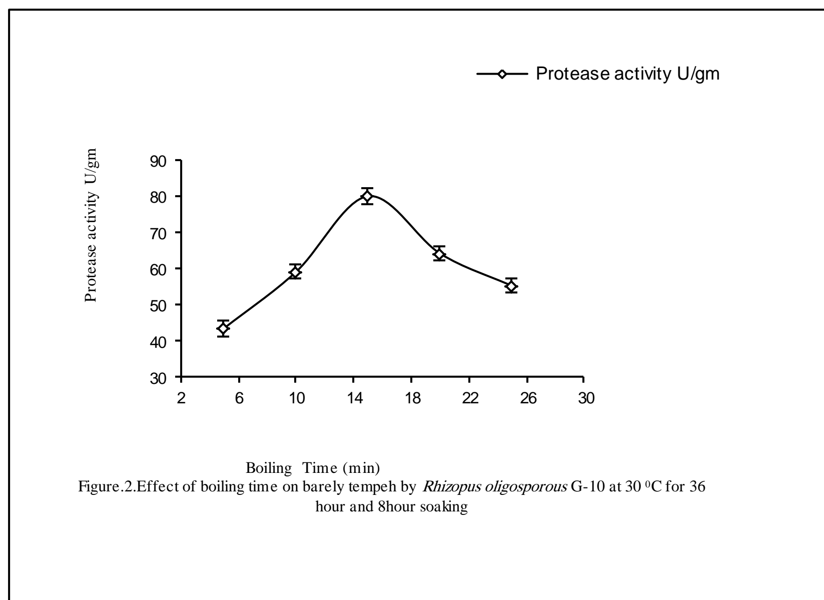

Figure.2.Effect of boiling time on barely tempeh by *Rhizopus oligosporous* G-10 at 30 °C for 36 hour and 8hour soaking

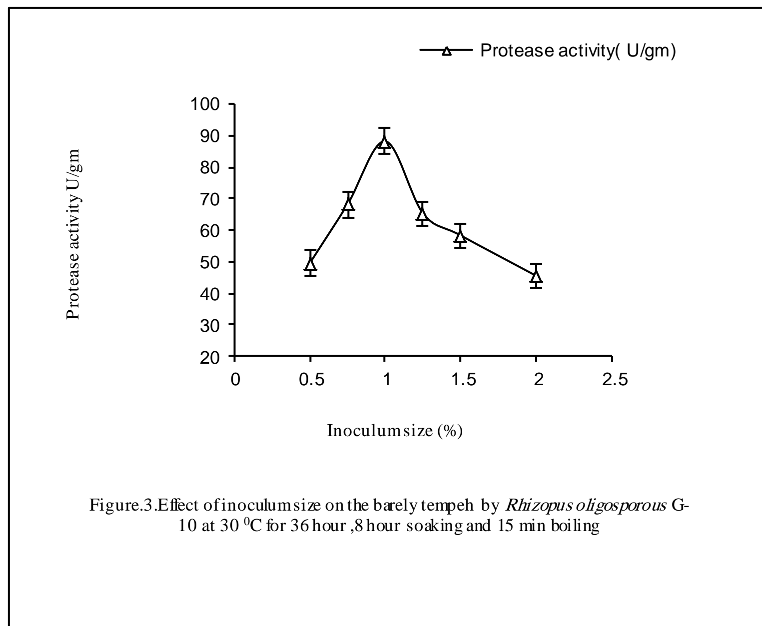

Figure.3.Effect of inoculumsize on the barely tempeh by *Rhizopus oligosporous* G-10 at 30 ^{0}C for 36 hour ,8 hour soaking and 15 min boiling

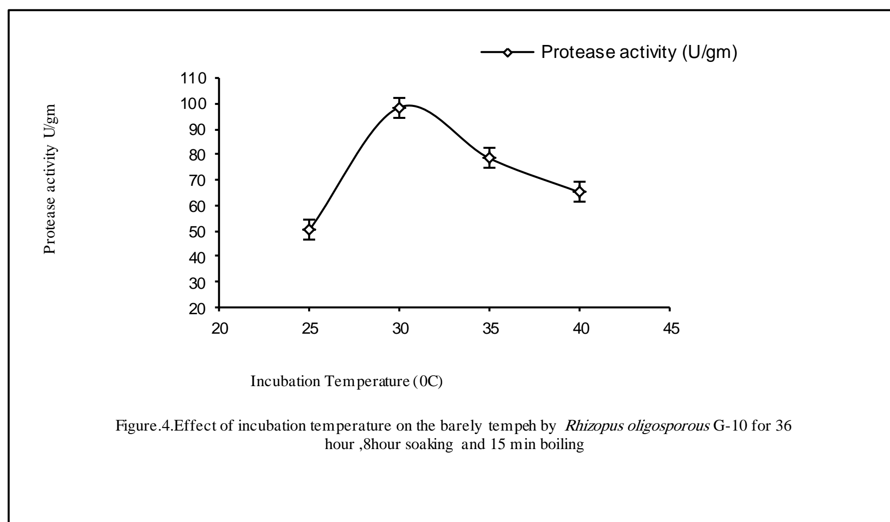

Figure.4.Effect of incubation temperature on the barely tempeh by *Rhizopus oligosporous* G-10 for 36 hour ,8hour soaking and 15 min boiling

Table 1.In vitro protein digestibility of fermented and unfermented barley. Values are the average of three replicates. ±denotes the standard deviation among triplicates.

Time (minute)	pH	*In vitro* Protein Digestibility (%)	
		Unfermented Barley Flour	Fermented Barley Flour
10	7.0	24.45 ± 0.45	48.36 ± 0.55
	7.2	25.32 ± 0.47	49.65 ± 0.54
	7.4	25.67 ± 0.50	50.85 ± 0.57
	7.6	26.35 ± 0.49	52.35 ± 0.58
	7.8	27.85 ± 0.48	57.98 ± 0.58
	8.0	28.55 ± 0.55	62.56 ± 0.75
	8.2	26.24 ± 0.48	54.48 ± 0.59
	8..4	25.26 ± 0.34	47.67 ± 0.47
	8.6	24.71 ± 0.32	45.65 ± 0.46
15	7.0	24.35 ± 0.45	46.36 ± 0.48
	7.2	24.82 ± 0.46	48.67 ± 0.52
	7.4	25.07 ± 0.45	48.54 ± 0.56
	7.6	25.75 ± 0.48	51.55 ± 0.53
	7.8	26.75 ± 0.44	54.95 ± 0.54
	8.0	27.50 ± 0.51	56.64 ± 0.65
	8.2	26.04 ± 0.38	51.48 ± 0.50
	8..4	25.06 ± 0.32	45.57 ± 0.44
	8.6	24.11 ± 0.30	43.65 ± 0.42
20	7.0	23.12 ± 0.35	44.36 ± 0.43
	7.2	24.10 ± 0.40	46.35 ± 0.50
	7.4	24.47 ± 0.45	47.57 ± 0.52
	7.6	24.95 ± 0.41	48.51 ± 0.51
	7.8	25.55 ± 0.44	50.65 ± 0.53
	8.0	26.70 ± 0.50	54.44 ± 0.56
	8.2	26.04 ± 0.38	51.48 ± 0.50
	8..4	24.56 ± 0.31	45.07 ± 0.42
	8.6	24.01 ± 0.30	43.15 ± 0.40

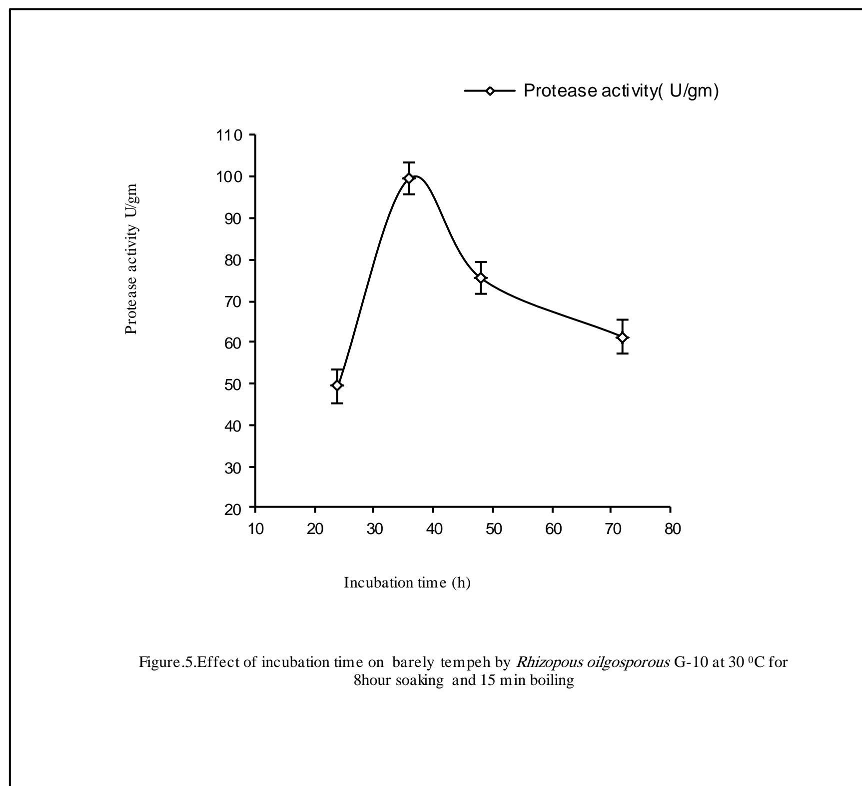

Figure.5.Effect of incubation time on barely tempeh by *Rhizopous oilgosporous* G-10 at 30 ⁰C for 8hour soaking and 15 min boiling

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