In Vitro DIGESTIBILITY EVALUATION OF RICE BRAN MEAL INCORPORATED WITH PRECIPITATE p-D-MANNANASE FROM *Penicillium italicum*

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Abstract: The effect of addition of crude and ammonium sulphate ((NH^SQi) precipitation of P-D-mannanase produced by *Penicillium italicum* on *in vitro* dry matter digestibility (IVDMD) and *in vitro* protein digestibility (IVPD) of rice bran meal intended for animal feed formulation was studied. Firstly, the crude enzyme (non-precipitated), and $(NH_4)_2SO_4$ precipitated enzyme were incorporated in the series of enzymes activities. Secondly, the effect of p-D-mannanase preparation and incubation time was evaluated on IVDMD and IVPD. Crude enzyme produced in basal medium supplemented with rice bran meal as the carbon source was assayed by dinitrosalicylic acid method and precipitated from culture supernatant with 70% ammonium sulphate concentration. Measurement of IVDMD and IVPD were carried out using pepsin-pancreatine assay. Results from statistical analysis showed significant interaction between the kind of enzyme preparations and activities on IVPD and IVDMD. *In vitro* protein digestibility (IVPD) of rice bran meal prepared with different enzyme preparations increased significantly ($P<0.05$) with increase in enzyme concentration. *In vitro* protein digestibility (IVPD) of rice bran meal prepared with ammonium sulphate increased all through the experiments with increase in enzyme concentration (0.1-0.5 mL), while decrease in protein digestibility was observed in rice bran meal prepared with crude mannanase beyond 0.4 mL enzyme supplementation. To study the effect of incubation time on digestibility of rice bran meal, 400 ul and 500 pi were used for crude and ammonium sulphate enzyme preparations respectively. IVPD of any enzyme additions (crude and ammonium sulphate enzyme preparations) at every incubation time was significantly above the non-enzyme treatment. Treatment with crude enzyme at 6 h incubation showed the highest IVPD, while ammonium sulphate precipitated enzyme at 8 h showed the highest IVPD. Incorporation of crude and ammonium sulphate precipitated enzyme improved the percentage IVDMD of rice bran meal all through the fermentation time. Incorporation of p~ D-mannanase might enhance the digestibility of ration containing high mannan, and ezyme precipitation with ammonium sulphate could be applied.

Key words: Penicillium italicum; in vitro dry matter digestibility; in vitro protein digestibility; rice bran meal; p-D-Mannanase.

INTRODUCTION

A potential for microbiologically modifying or enriching agricultural wastes as stock-feed has been inve: tigated extensively in the past (Ghaly et al., 2005; Stabnikova et al., 2005; Gélinas and Barrette, 2007; Ke et al., 2011). Microbial conversion, especially fungal bioconversion of wastes seems to be a practical and promising alternative for increasing their nutritional values, transforming them into animal feed and thus producing value-added products (Villas-Bôas et al., 2003; Agosin et al., 2006), fungal bioconversion of agricultural by-products is an environmentally friendly biotechnological process (Ke et al., 2011;

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Zhang et al., 2002; Mukherjee and Nandi, 2004). Filamentous fungi have been reported to biodegrade varieties of agricultural wastes through the secretion of extracellular enzymes (phytases, mannanases, xylanases, cellulases, tannanases, lipases, pectinases, proteases and so on (Akinyele and Agbro, 2008).

Feed efficiency of low quality poultry ration rich in mannan, one of the hemicelluloses can be enhanced by solid state fermentation process using mannolytic microbes such as *Streptomyces* sp., *Eupenicillium javanicum*, *Aspergillus niger* NRRL 337 (Purwadaria et al., 1995), and *Penicillium italicum* (Akinyele et al., 2013). Solid state fermentation could improve the *in vitro* and *in vivo* digestibility of low quality poultry meals (Purwadaria et al., 1995; Purwadaria and Haryati, 2003), however, this process is not favorable for poultry and fish feed mill industries. In the feed mill the enzymes are incorporated before pelleting process and catalyze the diet within the animal digestion system. Experiments have been conducted to use mannanases to improve the feed coefficient ratio of feeds containing rich mannan such as coconut meal (Purwadaria and Haryati, 2003; Khanongnuch et al., 2006) and guar gum (Ray et al., 1981) or common corn and soybean diets (Jackson. et al., 1999). To the best of our knowledge, there is no information on the pretreatment of rice bran meal with enzyme preparations.

In recent years, a β-D-mannanase produced from bacteria and fungi has been shown to improve feed conversion and performance to broiler, turkeys and swine (Khanongnuch et al., 2006). The important role of β-D-Mannanase is hydrolyzing β-1, 4-glycosidic linkages in β-mannan and can reverse the negative impact of caused by β-mannan. Without solid substrate fermentation treatment or hydrolytic enzyme addition, the fibre content limits rice bran meal feed efficiency in the diets. *P. italicum*, a mold isolated from yam peels (Akinyele et al., 2013; Arotupin and Olaniyi, 2013) could produce high β-D-mannanase under solid substrate fermentation enriched with mineral medium containing copra meal.

The activity of β-D-mannanase produced from *P. italicum* was greater than what was reported in the findings of El-Naggar et al. (2006) when *Aspergillus niger* was used as a mannanase producer. Precipitation either with ammonium sulphate or ethanol is a first step to concentrate and partially purify the enzyme (Purwadaria and Haryati, 2003). Different preparations will precipitate different protein types and concentrations that affect the hydrolytic activity. The activity of the precipitated enzymes to digest high mannan rations could be evaluated through *in vitro* or *in vivo* method (Purwadaria and Haryati, 2003). The purpose of this experiment was to evaluate *in vitro* dry matter and protein digestibilities of rice bran meal incorporated with precipitated β-D-mannanase from *P. italicum*.

MATERIALS AND METHODS

Fungal Isolate

Penicillium italicum isolated from agro-wastes previously confirmed positive for mannanase activity by plate assay in our previous work was used in this study. The fungal isolates were identified in the Microbiology Research Laboratory, Federal University of Technology, Akure, Ondo State, Nigeria according to the method designed by Pitt ad Hocking (1997) on the bases of cultural characters (colour, shape of colony, surface and reverse pigmentation and texture of the colony) as well as microscopic structure (septate or nonseptate hyphae, structure of hyphae and conidia). The fungal isolate was maintained on Locust Bean Gum (LBG) containing agar plates and sub-cultured at regular intervals. They were incubated at 30±2° C until the entire plates were covered by active mycelium and stored at 4°C in refrigerator on agar slants.

Chemicals and Substrates

Rice bran utilized as substrate for fermentation was procured from a registered rice mill in Akure, Ondo State, Nigeria. The substrate was washed, sun and oven-dried at 70°C with a Heating Drying Oven (Model DHG, England) for a period of 2 h, sieved to 40 mm mesh size and stored in air tight transparent plastic containers to keep it moisture free (Akinyele *et al.*, 2013). Locust Bean Gum (LBG) was purchased from Sigma Chemicals (St. Louis, MO). All other chemicals were of analytical grade.

Media Preparation and Enzyme Production

For the production of mannanase in submerged state fermentation, the isolate was grown at 30°C in 250 mL Erlenmeyer flask in Mandels and Weber's medium modified by Iqbal *et al.* (2010). This medium contained the following ingredients (g/L): Rice bran 10, Peptone 2, yeast extract 2, NaNO3 2, K2HPO4 1, MgSO4.7H2O 0.5, KCl 0.5 and FeSO4.7H2O traces. Final pH was adjusted to 6.0 and then sterilized at 121°C for 15 min. After sterilization, each flask was inoculated with two discs of 8 mm diameter of the organism from mannan-containing agar medium using sterile cup borer (Arotupin and Olaniyi, 2013). The flasks were incubated at 30°C for 5 days at static condition. Sterile medium supplemented with rice bran meal without organism served as the control.

Enzyme Extraction and Preparation

The solid materials and fungal biomass were separated by centrifugation at 6000 rpm for 15 min at 4°C using refrigerated centrifuge (Centurion Scientific Limited). The clear supernatant was used for enzyme assays and soluble protein determination. The supernatant (100 mL) obtained after centrifugation was precipitated by adding 47.2 grams to ammonium sulphate to achieve 70% ammonium sulphate concentration. Each treatment was carried out in triplicates and the results obtained throughout the work were the arithmetic mean of at least 3 experiments.

Enzyme Assays

Mannanase activity was assayed in the reaction mixture composing of 0.5 mL of 50mM potassium phosphate buffer pH 7.0 and 1% LBG with 0.5 mL of supernatant at 45°C for 60 min modified method of Arotupin and Olaniyi (2013). Amount of reducing sugar released was determined by the dinitrosalicylic acid reagent (DNS) (Miller, 1959). One unit of mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

Determination of IVDMD and IVPD

IVDMD and IVPD of rice bran meal were determined according to pepsin-pancreatin method (Lowry et al., 1951). The digestion was carried out on 250 mg rice bran meal with 15 mL of pepsin (Sigma) 0.1% in HCl 0.1 M for 3 h at 40°C and followed by neutralization with NaOH 0.5 N, and further digestion with 7.5 mL phosphate buffer (8.0) containing 4 mg pancreatin (Sigma) for 24 h at 40 °C. IVDMD was determined only by gravimetry after drying the residues at 105°C, while IVPD was followed by determination of the protein content in the residues (Lowry et al., 1951).

Experimental Designs

Evaluation of β-D-mannanase addition on IVDMD and IVPD of rice bran meal was carried out in two successive experiments. Firstly, the crude enzyme (non-precipitated) and $(NH_4)_2SO_4$ precipitated enzyme were incorporated in the series of enzymes activities stated as volumes: 0.1, 0.2, 0.3, 0.4 and 0.5 mL. The enzyme addition was carried out at the same time at pancreatin addition. The dry matter and protein residue were determined after 24 h incubation of pancreatin and β-D-mannanase addition. Secondly, the effect of different β-D-mannanase preparations and incubation

time at different time intervals (0, 2, 4, 6 & 8 h) on IVDMD and IVPD were evaluated.

Statistical Analysis

Data presented on the average of three replicates (± SE) are obtained from their independent experiments. Experiment data was subjected to ANOVA of SPSS programming. Duncan's multiple range tests was used to identify significant differences between means of treatments.

Results

Effect of Different Types of Mannanase Preparations and Activities on *In vitro* Protein Digestibility of Rice Bran Meal

Data obtained from IVPD showed that there was significant interaction between type of enzyme preparations and β-D-mannanase volume addition (Table 1). IVPD of rice bran meal prepared with different enzyme preparations increased significantly ($P<0.05$) with increase in enzyme concentration. *In vitro* protein digestibility (IVPD) of rice bran meal prepared with ammonium sulphate increased all through the experiments with increase in enzyme concentration (0.1-0.5 mL), while decrease in protein digestibility was observed in rice bran meal prepared with crude mannanase beyond 0.4 mL enzyme supplementation. Therefore, for further experiments on the determination of incubation time, 500µl (0.5 mL) was used for ammonium sulphate enzyme preparation, while 400µl (0.4 mL) of enzyme addition was used for crude enzyme preparation.

Table 1: Effect of different types of mannanase preparations and activities on IVPD of rice bran meal

Conc. (mL)	(Crude) Protein (mg/mL)	(NH4SO4) Protein (mg/mL)
0.1	7.537^{b}±0.00	8.301^{a}±0.02
0.2	9.486^{c}±0.00	10.042^{b}±0.00
0.3	11.032^{d}±0.00	12.667^{c}±0.01
0.4	12.833^{e}±0.00	14.464^{d}±0.01
0.5	1.000^{a}±0.01	21.018^{e}±0.00

Activity of each type of prepared mannanase was adjusted to 43.5 U/mL, Means with the same superscript letters along the same row are not significantly different ($P<0.05$).

Effect of Incubation Time on *In Vitro* Protein Digestibility of Rice Bran Meal after Addition of Different Types of Mannanase Preparations

IVPD of any enzyme additions at every incubation time was significantly above the non-enzyme treatment. Treatment with crude enzyme at 6 h incubation showed the highest IVPD, while ammonium sulphate precipitated enzyme at 8 h showed the highest IVPD (Table 2). The values of IVPD were increasing in the course of incubation time. Significant interaction ($P<0.05$) between type of enzyme preparations and incubation time appeared. In every incubation time IVPD of samples with any enzyme addition were significantly higher than the control (Time 0).

Table 2: Effect of incubation time on IVPD of rice bran meal after addition of different types of mannanase preparations

Hours	(Crude) Protein (mg/mL)	(NH4S04) Protein (mg/mL)
0	$0.329^{a}\pm0.01$	$0.394^{a}\pm0.00$
2	$2.176^{c}\pm0.00$	$0.370^{b}\pm0.00$
4	$1.856^{b}\pm0.00$	$8.287^{c}\pm0.00$
6	$3.056^{e}\pm0.01$	$9.446^{d}\pm0.00$
8	$2.778^{d}\pm0.01$	$9.861^{e}\pm0.00$

Activity of each type of prepared mannanase was adjusted to 43.5 U/mL, Means with the same superscript letters along the same row are not significantly different ($P<0.05$).

Effect of Incubation Time on the Percentage (%) *In Vitro* Dry Matter Digestibility of Rice Bran Meal after Addition of Different Types of Mannanase Preparations

The effect of enzyme additions at different incubation times on percentage dry matter digestibility of rice bran meal is revealed in table 3. The values of IVDMD from both enzyme preparations increased significantly all through the period of incubation. The value obtained for IVDMD after addition of crude enzyme and $(NH_4)_2SO_4$ enzyme preparation on rice bran meal at 8 h of incubation was 3.63 fold and 4.32 fold higher than IVDMD obtained from the corresponding control sample.

Table 3: Effect of incubation time on the percentage (%) IVDMD of rice bran meal after addition of different types of mannanase preparations

Hours (mg/mL)	(Crude) Protein (mg/mL)	(NH4SO4) Protein
0	$9.507^{a}\pm0.01$	$11.000^{a}\pm0.06$
2	$14.043^{b}\pm0.15$	$17.023^{b}\pm0.15$
4	$19.040^{c}\pm0.11$	$21.493^{c}\pm0.04$
6	$28.507^{d}\pm0.19$	$35.633^{d}\pm0.33$
8	$34.497^{e}\pm0.05$	$47.500^{e}\pm0.02$

Activity of each type of prepared mannanase was adjusted to 43.5 U/mL, Means with the same superscript letters along the same row are not significantly different ($P<0.05$).

DISCUSSION

β-D-mannanase hydrolyzed the mannan complex (hemicelluloses) of rice bran meal and resulted in the increase of *in vitro* dry matter digestibility (IVDMD). The enzyme might also contain low activities of cellulases and glycosidases (Purwadaria and Haryati, 2003). These enzyme mixtures synergistically took part in the fibre digestion, which then exposed the protein molecules previously protected by hemicelluloses and cellulose to be further digested by pepsin-pancreatin and thus, enhanced the *in vitro* protein digestibility (IVPD).

The increase in activity (higher enzyme volume) significantly enhanced digestibilities, thus 0.4 and 0.5 mL are considered optimal for crude and $(NH_4)_2SO_4$ enzyme preparations respectively to digest mannan in rice bran meal. The addition of 0.1 mL $(NH_4)_2SO_4$ enzyme preparation was considered enough for the digestion of coconut meal (Purwadaria and Haryati, 2003). The addition of cellulase activities did not increase the digestion of bagasse after optimal enzyme concentration (Manonmani and Sreekantiah, 1987). The increase of digestion activity occurred if the substrate was also increased. The precipitation treatment did affect the digestion of rice

bran meal better; this showed that precipitation with ammonium sulphate could be used in place of crude enzyme preparation.

Types of enzyme preparation influenced percentage IVDMD at all the times of incubation. These data showed that different enzyme preparations might produce different enzyme components. It is possible that ammonium sulphate precipitated enzyme digested more hemicelluloses that protect protein molecules or this could be related to protease activity from the mold enzyme. These data indicated that the enzyme could effectively improve the digestion of rice bran meal including other rich mannan ration in the condition of fish and poultry digestion system in a short time.

CONCLUSION

This experiment is the preliminary study for further *in vivo* trial. This study showed β-D-mannanase causes positive effect of digestibility of rice bran meal and that precipitated enzyme could be applied. Precipitation using ammonium sulphate is more appropriate than crude enzyme in term of economical aspect of application.

COMPETING INTEREST

Authors have declared that no competing interests exist.

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