Chemical Properties of Mannan Containing Polysaccharides from Palm Kernel Meal and Its Inhibitory Effect againts *Salmonella* spp and *E. coli*

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ABSTRACT

Mannan linkages can not be degraded by monogastric animals. Mannans, however, were proved to be useful in inhibiting the multiplication of pathogenic bacteria in the digestive tract of animals. The aim of this study was to investigate the chemical properties of mannan containing polysaccharides from palm kernel meal (PKM) and their in vitro inhibitory effect against *Salmonella* spp and *E. coli*. The combination of chemical, physical and mechanical treatments was used to extract the cell wall of PKM. The extraction product was then autoclaved at 121° C for 15 minutes and centrifuged at 16 000 *g* for 15 minutes. The supernatant was separated chromatographically using gel sephadex G-50 (3 x 80 cm) in order to separate the carbohydrate fraction from protein. The carbohydrate fraction was evaporated and evaluated for its chemical and physical properties. An agglutination test was done by adding a suspension of 1 ml bacteria to 1 ml of mannan containing polysaccharides. The inhibitory effect of mannans on bacteria was tested by incubating bacteria in liquid media containing 0, 1000, 2000, 3000 and 4000 ppm mannan containing polysaccharides in terms of total sugar. The extraction results showed that 669,72 mg mannan containing polysaccharides could be isolated from 100 g PKM. The polysaccharides had brown colour, sugar flavour, were soluble in water and consisted of glucose, mannose and galactose in a ratio of 8:20:1. The addition of mannan containing polysaccharides to the bacterial test did not show a visual agglutination effect but there was an indication that the bacteria clustering was effected by mannan containing polysaccharides. The total number of *Salmonella* spp and *E.coli in vitro* decreased as the concentration of mannan containing polysaccharides in the media increased. From the results of these experiments it can be concluded that the polysaccharide fraction in PKM is dominated by mannans. There is an indication that these mannans are able to inhibit *Salmonella* spp and *E. coli in vitro.*

Key words: Palm kernel meal, mannan, physico-chemical properties, *Salmonella spp.*, and *E. coli*

INTRODUCTION

The usage of feed additives together with high quality feeding rations is essential to support optimal animal production in the poultry industry. The utilization of antibiotics has been proven to increase growth rates and control the multiplication of pathogenic bacteria in the animals digestive track. However, the use of antibiotics in the poultry industry has been reported to cause several problems such as antibiotic residues in animal products and bacterial resistance to the antibiotics used. Those problems may be avoided by the utilization of natural feed additives obtained from local sources which are saver to use and can produce healthier animal products compared to the utilization of antibiotics. One of the local products that has the potential to be used as a natural feed additive is palm kernel meal (PKM) because PKM contains high levels of mannan containing polysaccharides.

The utilization of PKM in poultry rations is still very low due to its high content of non-starch polysaccharides which are dominated by mannose linkages. However, mannose linkages can be used as feed additives due to their inhibition effects on pathogenic bacteria (Lyons, 1996; Power, 1997). The extraction of mannose linkages from PKM is necessary to produce a natural feed additive.

Mannose linkages can be obtained from *Saccharomyces cerevisiae* cell wall extraction as it has been reported by Lyons, 1997, but the data regarding the physical and chemical properties of mannose linkages from PKM are still limited. This paper will describe the isolation method and the physico-chemical properties of mannan containing polysaccharides extracted from PKM.

MATERIALS AND METHODS

The raw materials used in this experiment were i.e., PKM, growth medium containing 0.1% glucose, 0.6% peptone, 0.1% yeast extract, 0.5% NH_4NO_3, 0.05% $MgSO_4.7H_2O$, 0.05% KCl, 0.1% NH_4Cl, 0.001% $FeSO_4.7H2O$, 0.05% K_2HPO_4, and 2% Bacto Agar with optimum pH 6.7 (Ramli *et al.*, 1995) and D-glucose as standard. The equipments used consisted of a microscope and High Performance Liquid Chromatography (HPLC).

Isolation of mannan from PKM

The sample preparation for the analysis of the polysaccharide component from PKM was based on the method of Ramli (1994). Before extraction, the samples were separated physically based on particle size and density using a vibrator (test sieve) Restsch 5657 HAAN. The product containing the highest hemicellulose and the lowest solubility was then extracted to obtain the polysaccharides. This process was started by grinding the PKM particles with or without a glass fraction, and then phosphate buffer was added until it formed a soft paste. Six combinations of physical and chemical extraction methods have been employed: P1, grinding (control); P2 grinding with the addition of 0.05 N NaOH; P3, grinding with the addition of 0.1 N NaOH; P4, grinding with the addition of a glass fraction; P5, grinding with the addition of 0.05 N NaOH and a glass fraction; and P6, grinding with the addition of 0.1 N NaOH and a glass fraction. After the extraction, the material was autoclaved at 121° C for 15 minutes, centrifuged at 10000g for 15 minutes and the supernatant was collected. The obtained supernatant (glicoprotein) was freeze-dried to reduce the volume, then the total sugars were calculated (mg). The glicoprotein was then treated with 0.1 N NaOH and incubated for 24 hours at 37° C, followed by dialysis for 48 hours at 4° C to obtain dialysate. The dialysate was then analyzed for its sugar content. The extraction method that yielded the highest sugar content was selected for further trials.

Separation of the carbohydrate and protein fractions

The dialysate product (mg) was injected into the chromatography column with the dimension of 3 x 80 cm containing Sephadex gel G-50 with aquadest as solvent. This process was used to separate the carbohydrate (polysaccharide) fraction from the protein fraction. The polysaccharide fraction was then re-chromatographed in the same column until no protein fraction did appear.

Measurement of sugar component in the polysaccharide fraction

The polysaccharide fraction that had been separated from the protein fraction was then hydrolysed with 2 M TFA at 105° C for three hours, neutralized using ethyl acetate and freeze dried. The product was then evaluated for its sugar components using HPLC equipped with a P-NH_2 Carbohydrate Column.

Agglutination test (*in vitro*)

The agglutination test was conducted qualitatively by mixing 1 ml bacterial suspension with 1 ml of the mannan containing polysaccharide suspension (Spring *et al.*, 2000). The mixture was left for five minutes before the occurrence of agglutination was observed under the microscope. There were six *Salmonella* strains used, namely *Salmonella enteritidis*, *S. typhimurium*, *S pullorum*, *Salmonella sp.*, *S. nadar*, dan *Salmonella sp.* isolated from manure) and four strains of *E. coli*

Bacterial Growth Inhibition Test on Agar Medium

The growth inhibition activity of mannan containing polysaccharides was tested against *Salmonella* and *E. coli* by culturing the Salmonella on Salmonella and Shigela Agar, and by culturing *E. Coli* on agar nutrient medium. Paper discs containing different level of mannans (0, 1000, 2000, 3000, and 4000 ppm) were put on the agar medium and incubated overnight. The next day, the clearing zones around the disc were observed and the diameter was measured. The total population of *Salmonella* and *E. coli* was also measured in liquid medium where different levels of mannan containing polysaccharides were added as described above.

Chemical and Statistical Analysis

Total sugars were measured using the method of Dubois *et al.* (1956) with D-glucose as standard. Data were analysed using analysis of variance and further tested using least significant difference (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Isolation of Mannan Containing Polysaccharides from PKM

By using a vibrator the PKM was separated into seven different fractions according to particle size. The chemical composition and physical characteristics of the fractions are described in Table 1. The physical appearance (size and color) of each fraction was distinct. Particle size did not influence the chemical composition significantly with the exception of hemicellulose (HC) where a clear increase with increasing particle size could be demonstrated. The solubility test showed that the fractions smaller in particle size tended to have a lower solubility compared to fractions with a larger particle size. Density increased as the pore sizes of the sieve increased up to 0.600 mm but decreased again as the pore sizes were getting larger.

This screening method was important to obtain optimum mannan containing polysaccharides, whereas other fractions can be used as sources of good quality protein. A high yield of mannan containing polysaccharides was obtained from the fraction that contained high levels of hemicellulose (screen size 2360 mm), considering that the mannan component mainly exists in hemicellulose. Kennedy (1990) reported that hemicellulose was composed of mannose, glucose, xylose, arabinose, and galactose. As shown in Table 1, the fraction No. 2 is the fraction that contains the highest hemicellulose concentration, so that this fraction was used for further work.

Hemicellulose is an integral part of the PKM cell wall, therefore in order to isolate hemicellulose it is not sufficient to only grind the material, but it requires a treatment that is able to degrade the cell walls. Using a glass fraction and/or glass fraction+NaOH for the PKM extraction process showed better yields compared to a procedure without the glass fraction. The total sugar content of PKM extracted using a glass fraction and/or glass fraction+NaOH was 1.5-2 times higher than that extracted without the glass fraction (Table 2). The extraction with NaOH without the glass fraction was not effective in increasing the amount of polysaccharides isolated from PKM. This indicated that PKM cell walls were difficult to be degraded using only 0.05 – 0.1 N NaOH. The combination of the glass fraction and 0.05 N NaOH significantly ($P<0.05$) increased the sugar content of the extract (Table 2), and the mannan content (Table 3). In contrary, the total sugar yielded from PKM extracted with the combination of the glass fraction and 0.1 N NaOH was significantly lower ($P<0.05$) compared to the PKM extracted by the glass fraction alone. This lower sugar content might be the result of high concentrations of NaOH destroying some sugar components in the polysaccharide. Therefore, the extraction method using the combination of 0.05 N NaOH and the glass fraction (P5) was used for further experiments.

The sugar component of the PKM polysaccharides composed of glucose, mannose, and galactose. The concentration of those sugars varied greatly depending on the treatments used. In general, the sugar linkages in the hemicellulose fraction (glucose, mannose, and galactose) of PKM consisted of alpha and betha linkages. Galactose and mannose are composed of alpha linkages which are easier degradeable. This was demonstrated in this experiment by the high sugar monomers ratios when the PKM was ground using aquadest (P1 in Table 3). The ratio declined when PKM was ground using NaOH (P2 and P3 in Table 3) as existing sugar monomers might become destroyed. The mannose consisting of betha linkages was found to be higher in PKM compared to galactose. This is documented by the increase of the mannose component compared to the galactose component when NaOH was used at a concentration of 0.05 in the extraction process (P2 and P5 in Table 3). These results show that that galactose linkages in polysaccharides from PKM are not as stable as mannose linkages. Ramli (1995) reported that cell walls from *Fusarium sp* contained mannose linkages resistant to chemical treatment.

Mannan containing polysaccharides from PKM as well as from *Penicillium spp* have brown colour, sugar smell, and are soluble in water. The polysaccharide profile using Sephadex gel G-50 for the separation is presented in Picture 1 and 2. Polysaccharides isolated from PKM by using the glass fraction procedure had more peaks compared to the extraction without the glass fraction. This result supports the previous result, which showed that the extraction with the glass fraction was more efficient in degrading cell walls of PKM. The number of peaks indicates the diversity of molecular weight of the extracted polysaccharides. High molecular weight polysaccharides are easier to be purified compared to low molecular weight polysaccharides because there is less protein impurities in high molecular weight polysaccharide.

The total sugar from PKM polysaccharides was 2.2 times higher than from *Penicillium spp* polysaccharides as reported by Ramli (2000). Both polysaccharide sources were composed of glucose, mannose, and galactose monomers in a the ratio of 8:20:1 in polysaccharides from PKM and in a ratio of 11:15:1 in polysaccharides from *Penicillium spp*. The mannose content was equivalent to 68.9% in PKM and to 55.5% in *Penicillium spp*. Daud *et al.* (1993) reported that the percentage of mannose was 56.4% in polysaccharides from

PKM. Based on the data shown above and considering the availability of PKM in Indonesia, it seems to be more efficient to extract mannan containing polysaccharide from PKM instead of using *Penicillium spp.* In addition, the use of PKM as feed additive for animals will raise less concerns compared to the usage of *Penicillium spp.* Based on these findings and the potential antimicrobial properties, mannan containing polysaccharides from PKM were tested for their activity against pathogenic bacteria in the digestive tract of poultry.

AGGLUTINATION TEST

The results of the agglutination test using several strains of *Salmonella* spp and *E. coli* showed that mannan containing polysaccharides visually did not show an agglutination effect, but microscopically it appeared that there were differences between the control treatment and the treatments including mannan containing polysaccharides. The bacteria appeared in clusters when the suspension was treated with mannan containing polysaccharides, whereas in the control treatment the bacteria spread evenly on the microscope slides (see Picture 3). This observation gives an indication that mannan containing polysaccharides from PKM have the ability to bind both *Salmonella* and *E. Coli*. This result is supported by previous experimental result reported by Lyons (1996) and Power (1997) showing that mannan oligosaccharide (MOS) isolated from cell walls of *Saccharomyces cereviceae* had an antibacterial activity. In addition, Turner *et al.* (2000) reported similar effects against *Salmonella thypimurium*, and Ishihara *et al.* (2000) found effects against *Salmonella enteritidis*. The antimicrobial activity of mannan containing polysaccharides isolated from PKM have never been reported before, so that the result of this experiment contain novel information related to the potential use of mannan containing polysaccharides from PKM as a feed additive with antimicrobial properties..

BACTERIAL INHIBITION TEST

The bacterial inhibition test was conducted using an agar plate as a medium as well as using a liquid medium. Both test media contained different concentrations of mannan containing polysacharides. The bacterial inhibion was measured by the clearing zone on the agar plate or in the liquid medium. The agar plate test showed slightly difference between the control group and the different treatments. In contradiction to these results, the test using the liquid medium showed a significance inhibition of the bacterial population. The reduction of the bacterial population was linear with the increasing content of mannan containing polyschararides from PKM. The result are shown on Picture 4.

The clearing zone test did not show a response indicating that mannan containing polysaccharides did not have a bactericide effect on this media but could have a bacteriostatic effect. Therefore the mode of action of mannan containing polysaccharides should be different compared to antibiotics. There is indicate that MOS inhibited the colonization of pathogenic bacteria (*Salmonella, E. coli,* and *Vibrio cholera*) on the surface of the digestive tract by binding bacterial lectin and avoid the attachment of the bacteria to the surface of the gut wall. In addition, MOS can be used as a carbon source for beneficial bacteria such as bifidobacteria.

The bacterial growth inhibition test using the liquid medium showed that there was a significant inhibition effect by mannan containing polysaccharides. The inhibition effect of the mannan containing polysaccharides was proportional to their level in the medium resulting in a dose dependent growth inhibition. This inhibition effect can be explained by a partial attachment of the bacterial cell walls to the mannan containing polysaccharides which could reduce the bacterial multiplication. Similar result were reported by Bailey (1991) who tested the ability of fructooligosaccharides (FOS) inhibiting the colonization of *Salmonella spp.*

The results of these experiments give an indication that mannan containing polysaccharides isolated from PKM can have a potential benefit in controling pathogenic bacteria in animal production. However, these results are so far only based on in vitro experiments and need to be validated in vivo. Therefore the investigation of mannan containing polysaccharides isolated from PKM in broilers and layers are in progress.

CONCLUSION

The combination of chemical and physical extraction methods using 0.05 NaOH and a glass fraction (P5) are good extraction methods to obtain mannan containing polysaccharides from PKM. An agglutination test showed that the treatment with mannan containing polysaccharides resulted in a clustering of bacterial such as Salmonella or E. Coli indicating that these polysaccharides have the ability to bind bacteria. A bacterial inhibition test using an agar plate as a media shown a slightly inhibition effect on the tested bacteria strains.

However, using a liquid media mannan containing polysaccharides from PKM dose dependently reduced bacterial growth. These results indicate that polysaccharides are able to inhibit the growth of *Salmonella spp.* and *E. coli in vitro*. Further experiments are necessary to validate this hypothesis in vivo.

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Tabel 1. Physical and Chemical Properties of PKM Fractions Screened by Using a Vibrator

No	Pore size of screen (mm)	Chemical composition (% DM)					Physical properties	
		CP	CF	NDF	ADF	HC	Solubility (%)	Density (g/cm^3)
1.	4,750	15,7	44,1	70,5	63,8	6,68	21,1	0,380
2.	2,360	16,3	42,0	72,3	48,8	23,5	21,2	0,403
3.	1,180	17,9	39,2	65,4	58,4	7,00	17,3	0,409
4.	0,600	15,1	44,0	71,8	67,7	4,12	16,9	0,496
5.	0,300	16,1	42,2	66,2	61,4	4,8	17,8	0,450
6.	0,150	15,3	38,2	70,2	68,2	2,0	19,4	0,400
7.	0,038	15,0	38,0	72,0	70,5	1,5	18,0	0,350

Note: DM: dry matter; CP: crude protein; CF: crude fiber; NDF: neutral detergent fiber; ADF: acid detergent fiber; HC: hemicellulose

Tabel 2. The content of total sugar, protein and crude fiber in PKM extract

Treatment	Total Sugar (mg/100g)	By-product of extraction (%)		Main product of extraction (%)		Nutrient extracted (%)	
		CP	CF	CP	CF	CP	CF
Control	-	14,84	40,95	-	-	-	-
P1	1438,80 ^{b}	14,78	34,84	0,05	6,11	0,36	14,92
P2	1209,05 ^{a}	15,51	36,99	0,00	3,96	0,00	9,68
P3	1262,20 ^{a}	14,96	40,23	0,00	0,72	0,00	1,77
P4	2240,73 ^{c}	7,42	18,42	7,42	22,53	49,99	55,01
P5	3294,70 ^{d}	7,83	18,02	7,01	22,93	47,23	56,00
P6	1264,52 ^{a}	8,52	18,10	6,32	22,85	42,60	55,81

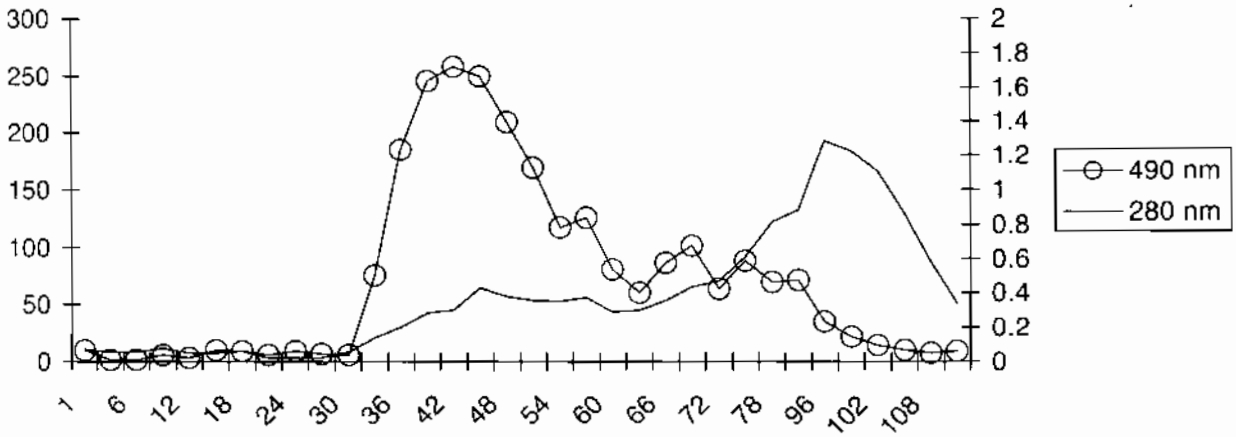
Note: CP: crude protein; CF: crude fiber; * different superscript in the same column means significantly different (P<0,05); P1=Aquadest; P2= NaOH 0,05 N; P3 = NaOH 0,1 N; P4 = Aquadest + glass fraction; P5 = NaOH 0,05 N + glass fraction; P6 = NaOH 0,1 N + glass fraction

Table 3. Ratio of sugar components in PKM polysaccharides using HPLC detection

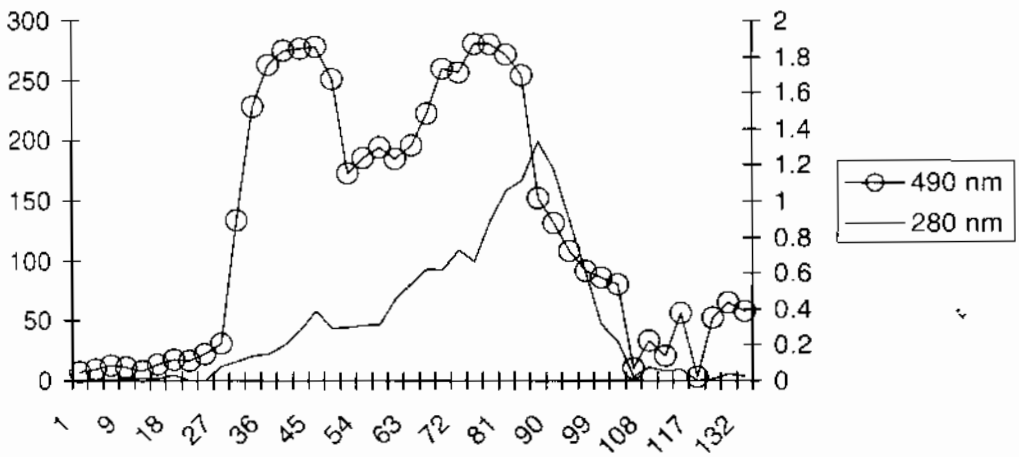
Treatment	Sugar component		
	Galactose	Glucose	Mannose
P1	13	1	14
P2	9	1	10
P3	14	1	2
P4	5	1	17
P5	8	1	20
P6	8	1	14

Note : P1 = Aquadest; P2 = NaOH 0,05 N; P3 = NaOH 0,1 N P4 = Aquadest + glass fraction; P5 = NaOH 0,05 N + glass fraction; P6 = NaOH 0,1 N + glass fraction

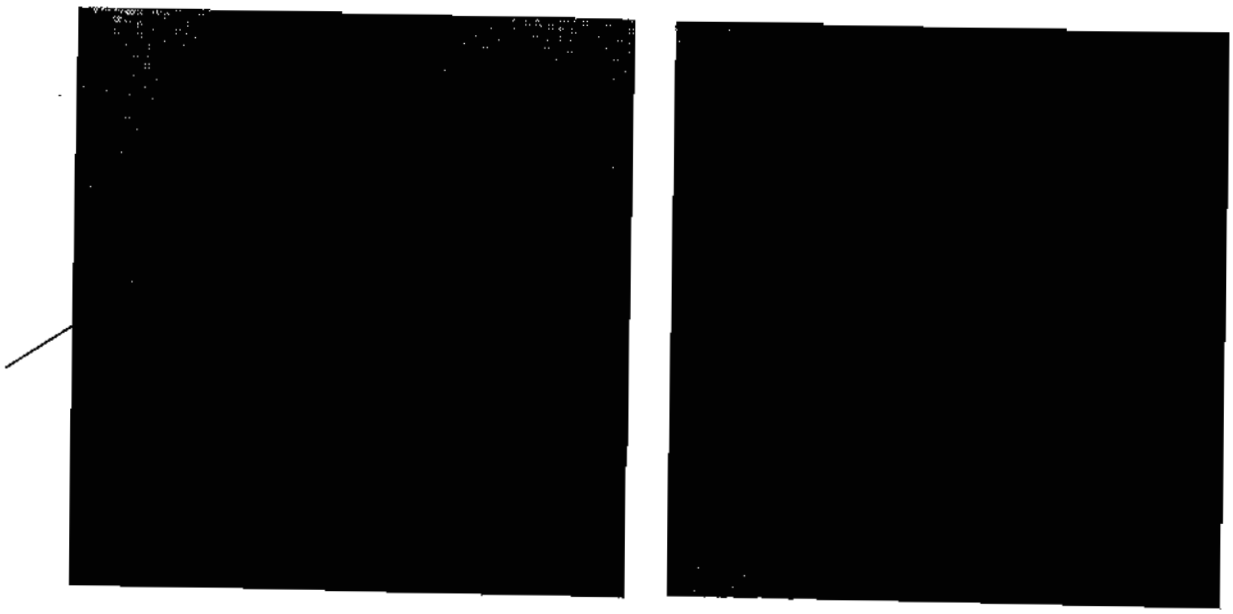
Picture 1. **Separation of PKM polysaccharides without a treatment using Sephadex G-50**



Picture 2. **Separation of PKM polysaccharide with a glass fraction using Sephadex G-50**



Picture 3 Agglutination Test against *Salmonella enteritidis*



PKM Extract

Control

Picture 4 The effect of mannan containing polysaccharides (ppm) on the bacterial population (log CFU)