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Pakistan Journal of Nutrition 9 (5): 471-474, 2010 ISSN 1680-5194 © Asian Network for Scientific Information, 2010 Isolation, Characterization and Production of Phytase from Endophytic Fungus its Application for Feed Yetti Marlida1, Rina Delfita2, Peri Adnadi2 and Gita Ciptaan1 1Department of Animal Nutrition, Faculty of Animal Science, Andalas University, Padang, Indonesia 2Department of Biology, Andalas University, Padang, Indonesia Abstract: Thirty four isolates of endophytic fungus produce phytases were isolated from leaf, stem and root fragments of soybean. Two isolates were the best of phytases enzyme producer and identified as Rhizoctonia sp. and Fusarium verticillioides. The phytase production was induced by phytate in medium used. The crude preparations were used in subsequent characterization studies, pH and temperature optimum and compared to other phytases tested and is thus a promising candidate for animal feed applications. The results showed that optimal production of phytase from Rhizoctonia sp. were pH 4.0 and temperature 50oC and pH 5.0, temperature 50oC for Fusarium verticillioides. Key words: Endophytic fungus, phytase, soybean, Rhizoctonia sp., Fusarium verticillioides INTRODUCTION Phytate (myo-inositol-hexaphosphate) is the major form of phosphorus stored in cereals, pollens, legumes and oil seeds. Phytate is known as an anti-nutrient factor, since it chelates various metal ions such as Mg+2, Ca+2, Zn+2, Fe+2, Fe+3 and forms complex with proteins (Pallauf and Rimbach, 1996; Martin et al., 2005; Cao et al., 2007; Liu et al., 2007). Morever, phytate is not metabolized by monogastric animals, which have low levels phytate- degrading enzymes in their digestive

tracts, Thereby, inorganic phosphate has to be added to feeds to ensure a sufficient phosphate supply for these animals. Consequently, the phytate in animal feeds is discharged continuing to rise (Vats and Banerjee, 2004). In Indonesia, the phytase has been new research. The possibility of using these phytases in industry has not investigated. However, more work needs to be done to obtain superior enzyme for industrial applications. It includes screening for strain that produce high phytase with better physicochemical properties, including high thermostability and suitable pH, along with gen cloning. The objectives of this study were to isolate, characterization production of phytase enzyme from endophytic fungus and its applications for feed. MATERIALS AND METHODS in feces of these animals into waterways, which Calcium phytate was made in the laboratory by adding contributed to eutrophication for surface waters, phytic acid into a saturated calcium hydroxide solution. particularly in areas of livestock production (Takizawa, Sodium phytate and sodium dodecyl sulfate were 1998). sourced from Sigma. All other reagents were domestic One way to enhance phosphate utilization from phytate products of analytical grade. is the use of phytase. To obtain a good source of phytase, a variety of microorganisms, animals tissue Isolation of endophytic fungus: Isolation of phytase and plant have been screened for enzyme. Several plant producers was performed by the agar plate of method phytases in wheat, barley, bean, corn, soybean, rice and Quan et al. (2001). Leaf, stem and root fragments cotton have been studied extensively (Greiner and sample of soybean [Glycine max (L.) Merril] were Konietzny, 2006). Microbial sources are Bacillus sp. obtained from a farmer garden in Padang, Indonesia. All (Poward and Jagannathan, 1982), Eschericia coli leaf stem and root samples were washed twice in (Greiner et al., 1993a,b), Enterobacter (Yoon et al., 1996) distilled water then surface sterilized by immersion for 1 Raoutella sp. (Greiner et al., 1997; Shah and Parekh, minute in 70% (v/v) ethanol, 4 minutes in sodium 1990), Aspergillus niger, Aspergillus fumigatus, hypochlorite [3% (v/v) available chlorine] and 30 sec in Aspergillus terreus (Howson and Davis, 1983) and 70% (v/v) ethanol and then washed three times in ruminal bacteria (Yanke et al., 1998). Several types of sterilized distilled water for 1 min each time. After fungal phytase are available on the market from several surface sterilization, the samples were cut into 5-7 mm companies such as Gist Brocades Co, Novo Nordisk Co pieces and aseptically transferred to plates containing and Kyowa Hakko Kogyo Co (Takizawa, 1998). At the 0.1% Ca-phytate; 1.5% glucose; 0.2% $NH_4NO_3$; 0.05% end of 20th century, annual sales of phytase as feed KCl; 0.05% $MgSO_4 \cdot 7H_2O$; 0.03% $MnSO_4 \cdot 4H_2O$; 0.03% additive were estimated at US$ 500 million and are $FeSO_4 \cdot 7H_2O$ and 1,5% agar. The final pH was adjusted Corresponding Author: Yetti Marlida, Department of Animal Nutrition, Faculty of Animal Science, Andalas University, Padang, Indonesia to 5.5. Cultivation carried out at 28oC for 2-5 days. Fungal colonies, capable of hydrolyzing Ca-phytate which can be recognized by their surrounding clear halo, were selected and repeatedly streaked onto solid Potato Dextrose Agar (PDA) plates. Colonies which developed on the plates were inspected for their morphology. Pure colonies were obtained by replating single colonies. Identification of fungal phytase was determined with using of methods Samson and Van Reenen-Hoekstra (1988); Barnett and Hunter (1972). Screening of endophytic for phytase produser: Each of isolated strains was grown in 50 ml of liquid medium (0.1% Ca-phytate; 1.5% glucose; 0. 2% $NH_4NO_3$; 0.05% KCl; 0.05% $MgSO_4 \cdot 7H_2O$; 0.03% $MnSO_4 \cdot 4H_2O$; 0.03% $FeSO_4 \cdot 7H_2O$, pH 5.5) in 500-ml Sakaguchi flask and incubated at 28oC for 48 h on reciprocal shaker (200 rpm). Cells collected from 1 ml of culture by centrifugation at 5000 x g for 10 min in cood room (4oC). Then, the collected cells were resuspended in acetate buffer (0.2 M, pH 5.5) and used for the phytase activity assay. Measurement of enzymatic activity: The phytase activity assay was determined by measuring the amount of liberated inorganic phosphate

according to a method of Quan et al. (2001). Reaction mixture consisted of 0.8 ml acetate buffer (0.2 M, pH 5.5) containing I mMNa -phytate and 0.2 ml of cell suspension. After incubation for 30 min at 37oC, the reaction was stoped by adding 1 ml of trichloroacetic acid. A 1 ml aliquot was analyzed for inorganic phosphate liberated by method Kim and Lei (2005). One unit of enzyme activity was defined as the amount of enzyme liberating I nmol of inorganic phosphate per minute. Enzymatic characterization studies: The effect of the pH on the activity of phytase was examined from pH 2 .0-8.0 in 100 mM buffer. The buffers used were as follows: pH 1.0-3.5: Gly-HCl; pH 3.5-6.0: NaAc-NaOH; pH 6.0-7.0: Tris- HAc; pH 7.0-8.0: Tris-HCl. Temperature versus enzyme activity was measured over a range of 28-80oC. RESULTS Identification of isolates: A total 34 endophytic fungal strains were screened for their ability to produce extracellular phytase. Only two strains, forming clear peripheral zones on turbid agar plate, were isolated from root samples and their activities were determined using liquid culture. According to the results of morphological observation were classified as fungi. They are Rhizoctonia sp. and Fusarium verticillioides (Fig. 1). The changes of phytase activity in fermentation were shown in Fig. 2. The phytase activity of Rhizoctonia sp. was 0.77-2.72 U/ml and F. verticillioides was 0.79-6.11 U/ml. a A B b c Fig. 1: Morphology of endophytic fungal phytases. A-B = Rhizoctonia sp.; C-D = F. verticillioides; (100x); a = sclerotia; b = hifa; c = phialid; d = macroconidia Fig. 2: pH versus activity profiles of phytase from Rhizoctonia sp. and F. verticillioides Fig. 3: Temperature versus activity profiles of phytase from Rhizoctonia sp. and F. verticillioides Enzymatic characteristics: Phytase production in phytases also have an optimal range 4.0-5.0 (Cao et al., culture of Rhizoctonia sp. and F. verticillioides by 2007; Quan et al., 2001; Nakamura et al., 2000). The submerged fermentation reached its stationary growth phytase in both Rhizoctonia sp. and F. verticillioides phase after cultivation for 24 h. During exponential have pH 4.0 and 5.0 respectively and most stable at pH growth phase, its produced small amounts of phytase. range 2.0-7.0. were very compatible with the internal Phytase production occurred in late stage of the environment of monogastric animals' stomach such as exponential growth phase and the activity phytase in pigs and poultry. Compared to many other phytase increased gradually with increasing incubation time. producing strains which exhibit low enzyme activity at pH Both of enzyme activity increased abruptly and reached values associated with the upper digestive tract, the the maximal value of 0.46 U/ml and 0.77 U/ml Rhizoctonia sp. and F. verticillioides phytase activity is respectively. significantly higher, reaching levels of commercial acceptability. The optimum temperature of the these pH: The phytase activity of Rhizoctonia sp. reached the phytase did not reveal differences between phytase from highest point at pH 4.0 for 15 min and phytase activity of Aspergillus niger N-3 and Natuphos phytase, the latter F. verticillioides at pH 5.0 (Fig. 2). exhibiting maximum activity at 50oC (Martin et al., 2005). At present, a major drawback to the widespread use of Temperature: Both fungal phytase displayed maximum phytases in animal feed is the constraint of thermal activity were at temperature of 50oC (Fig. 3). At pH 8.0 stability required for these enzymes to withstand and at temperature 80oC almost all enzymatic activity of inactivation during the feed-pelleting or expansion both fungal were lost. processes (Cao et al., 2007). Both of phytases exhibited maximum activity as high as Natuphos and pGP209 DISCUSSION phytase, were at 50oC (Martin et al., 2005). This phytase Endophytic fungal phytase was isolated from leaf, stem reached levels of commercial acceptability. This phytase and root fragments sample of soybean and identified as is worthy of further research as retains activity over a Rhizoctonia sp. and F. verticillioides was identified for wide range of pH values characteristic of digestive tract further study. This is the first report of Rhizoctonia sp. and and could conceivably be more suited to increasingly F. verticillioides are extracellular phytase and exhibiting higher

feed processing temperatures currently employed high phytase activity. The phytase synthesis in in the animal feed industry. Rhizoctonia sp. and F. verticillioides by phytate in the culture medium. It may be concluded that only phytate ACKNOWLEDGEMENT induced these enzymes. Many other phytase producing This work was supported by Grant from DIKTI (Hibah strains as Eschericia coli, Pseodomonas sp and Bersaing). The authors are grateful to the laboratories Raoutella sp. also were induced by phytate (Shah and staff at Microbiology Lab. Universitas Andalas for their Parekh, 1990; Konietzny and Greiner, 2004). Enzyme support during the experiment. induction is due to physiological change in a whole microbial population and it involves an accelerated rate REFERENCES of renewed formation of enzyme in response to a Barnett, H.L. and B.B. Hunter, 1972. Ilustrated Genera of relatively specific nutritional stimulus (Rhodes and Imperfect Fungi. 3rd Edn. Burgess Publishing Fletcher, 1966). The phytase was induced early phase of Company. USA. cultivation. It seems that the phosphates are released Cao, L., W. Wang, C. Yang, Y. Yang, J. Diana, A. from phytate. Yakupitiyage, Z. Luo and D. Li, 2007. Application of Phytase often has a low-pH optimum range (pH 4.5-6.0) Microbial Phytase in Fish Feed. J. Enzyme and with a rapid drop in activity at pH value above 6.0. Yeast Microbial Technol., 40: 497-507. Greiner, R. and U. Konietzny, 2006. Phytase for Food Aplication. Food Technol. Biotechnol., 44: 125-140. Greiner, R., U. Konietzny and K.D. Jany, 1993a. Purification and Characterization of Two Phytases From Escherichia coli. Arch. Biochem. Biophys., 303: 107-113. Greiner, R., U. Konietzny and K.D. Jany, 1993b. Purification and Characterization of Two Phytases From Escherichia coli. Arch. Biochem. Biophys., 303: 107-113. Greiner, R., E. Haller, U. Konietzny and K.D. Jany, 1997. Purification and Characterization of A Phytase From Klebsiella Terrigena. Arch. Biochem. Biophys., 341: 201-206. Howson, S.J. and R.J. Davis, 1983. Production of Phytate-Hydrolysing Enzyme by Fungi. J. Enzyme Microbiol. Technol., 5: 377-382. Kim, T.W. and X.G. Lei, 2005. An Improved Method for a Rapid Determination of Phytase activity in animal feed. J. Anim. Sci., 83: 1062-1067. Konietzny, U. and R. Greiner, 2004. Bacterial Phytase: Potential Aplication, In vivo Function and Regulationof Its Synthesis. Braziliat. J. Microbiol., 35: 11-18. Liu, Z., H. Wang, E. Xiu Wang, H. Xu, D. Gao, G. Zhang, P. Chen and D. Liu, 2007. Effect of Wheat Perling on Flour Phytase Activity, Phytic Acid, Iron and Zinc Content. Food Sci. and Technol. Swiss. Martin, J.A., R.A. Murphy and R.F.F. Power, 2005. Purrification and Physico-Chemical Characterisation of Genetically Modifed Phytases Expressed in Aspergillus awamori. Bioresource Technol., 97. Irlandia. Nakamura, Y., H. Fukuhara and Sano, 2000. Secreted Phytase Activities of Yeast. Bioscience Biotechnol. and Biochem., 64: 841-844. Pallauf, J. and G. Rimbach, 1996. Nutritional Significance of Phytic Acid and Phytase. Arch. Anim. Nutr., 50: 301-319. Pimentel, I.C., C. Glienke-blanco, J. Gabardo, R.M. Stuard and J.L. Azevedo, 2006. Identification and Colonization of Endophytic Fungi from Soybean [Glycine max (L.) Merril] under Different Environmental Conditions. J. Brazilian Archieves of Biol. and Technol., 45: 705-711. Powar, V.K. and V. Jagannathan, 1982. Purfication and Properties of Phytate-Specific Phosphatase from Bacillus subtilis. J. Bacteriol., 151: 1102-1108. Quan, C., L. Zhang, Y. Wang and Y. Ohta, 2001. Prpduction of Phytase in Low Phosphate Medium by a Novel Yeast Candida krusei. J. Biosci. and Bioeng., 9: 154-160. Rhodes, A. and D.L. Fletcher, 1966. Principles of Industrial Microbiology. Pergamon Press. New York. Shah, V. and L.J. Parekh, 1990. Phytase From Klebsiella sp. No. Pg-2: Purification and Properties. Indian J. Biochem. Biophys., 27: 98-102. Samson, R.A. and E.S. van Reenen-Hoekstra, 1988. Intoduction to Food-Borne Fungi. Centraalbureau voor Schimmelcultures. Wageningen. Takizawa, N., 1998. Utilization of Recombinan Phytase on the Avoidance of Water Pollution by Phosporus of Excretory Organ of Liverstock. Seitbutsu-kakagu, 76. Vats, P. and U.C. Banerjee, 2004. Production Studies and Catalytic Properties of Phytase. Enzymes Technol. J., 35: 3-14. Yanke, L.J., H.D. Bae, L.B.

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