Evaluation and Isolation of Phytin Phosphohydrolyzing Bacterial Population in the Rumen

C. Suzuki and K. Ushida*

Laboratory of Animal Science, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan

ABSTRACT : A series of experiments was conducted to evaluate phytin phosphohydrolysis activity in the rumen and to isolate phytase positive rumen bacteria. Endogenous phytase activity of wheat bran was estimated and compared with that of bacterial phytin phosphohydrolysis. Substantial phytase activity was detected in wheat bran during *in vitro* rumen incubation. Bacterial phytase activity was suggested not to be high. Only two facultative anaerobes, *Klebsiella* sp. and *Corynebacterium* sp. were isolated as phytase producing organisms. These belonged to a minor microbial group in the rumen population. Protozoal fraction showed an initial velocity of phytin phosphohydrolysis 7 times higher than the bacterial fraction. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 7 : 957-961*)

Key Words : Phytin Phosphohydrolyzation, Dietary Phytase, Rumen Bacteria, Rumen Protozoa

INTRODUCTION

Development of intensive farming systems in the pig and poultry industry has increased excretion of phosphorus to the environment, since these animals cannot efficiently use plant phytin as a source of phosphorus (Khan, 1996). It is, therefore, of interest to improve phytin utilization in these animals by means of feed additives (Simons and Versteegh, 1990; Cromwell et al., 1995). The use of fungal phytase has been developed for this purpose and some of them have already been commercialized. Although phytase-producing probiotics seem to be another possibility to this end, there is no bacterial strain available at the moment. Ruminants have been regarded as efficient utilizers of plant phytin by means of rumen microflora (Reid and Franklin, 1947). However, little is known about phytase producing rumen microorganisms. In this experiment we tried to estimate the phytin-phosphohydrolyzing activity of rumen protozoal and bacterial fractions, and to enumerate and isolate these bacteria.

MATERIALS AND METHODS

Animals and diets

An adult Suffolk wether equipped with a rumen cannula and of 65 kg body weight was used as a donor of rumen fluid. It received a diet composed of 600 g timothy hay and 400 g commercial concentrate free from antibiotics (Rakunou Supporter 14, Kumiai Shiryo, Kobe, Japan) twice a day. Water and mineral block were always accessible to the animal.

* Address reprint request to K. Ushida. Tel/Fax: +81-75-703-5620, E-mail: k_ushida@kpu.ac.jp.

Received October 13, 1999; Accepted January 13, 2000

Estimation of dietary endogenous phytase activity

Commercially available wheat bran was obtained from Kumiai Shiryo. The bran was further milled and a portion (0.1 g) of it was suspended either in 3 ml cell-free rumen fluid (supernatant after centrifugation at 25,000 g for 15 min) or rumen bacterial fraction (supernatant after centrifugation at 150 g for 5 min). A portion of the milled bran was autoclaved (121°C 203 kPa) and used to prepare the suspensions as indicated above. Suspensions were incubated under O_2-free CO_2 at 37°C for 4 h. After incubation was completed, residual bran was recovered and washed by centrifugation (2,000×g for 5 min) in citrate buffer (0.3 M, pH 5.5). Washed residual bran was suspended in 3 ml citrate buffer containing 6 mg crude wheat phytase (P1259, Sigma, St. Louis, MO, USA) and incubated at 37°C for 4 h. At the end of incubation, bran was eliminated by centrifugation and free-phosphorus in supernatant was determined as described below. The amount of free phosphorus could give estimates of phytin contents of bran.

Phosphohydrolyzing activity of bacterial and protozoal fractions

Rumen fluid was sampled via a cannula 1 h after morning feeding. Sampled rumen fluid (200 ml) was squeezed through four layers of surgical gauze. Strained fluid was further subjected to a serial centrifugation. A protozoal fraction was prepared by low speed centrifugation (150×g for 5 min) and washed twice with citrate buffer (0.3 M, pH 5.5) and finally suspended in 200 ml of the same citrate buffer. Cells were disrupted by repeated freeze-thaw cycles at -20°C and room temperature. A crude enzyme preparation was made after elimination of cell debris at 25,000×g for 15 min. Crude enzyme was stored at -20℃ until analyses. A bacterial fraction was prepared

from the supernatant after low speed centrifugation. Supernatant was centrifuged at 25,000×g for 15 min and the pellet was washed twice with citrate buffer as described above, and finally suspended in 200 ml of the same buffer. Bacterial cells were disrupted in a crushed ice bath by ultrasonication (5 times of 50 W for 1 min: TOMY UD-200 Ultrasonic Disruptor) and cell debris was eliminated by centrifugation at 25,000 ×g for 15 min. Both fractions were stored at -20℃ until analyses. Phosphohydrolyzing activities of both cellular contents and cell wall fraction were determined separately, but these values were combined in figure 1 due to low activity. A portion (1 ml) of crude enzymes was mixed with the same volume of 10 mM sodium phytate solution (in citrate buffer, 0.3 M pH 5.5) and incubated at 37°C for 10, 30 and 60 min. At the end of incubation, 4 ml of trichloroacetic acid (5% w/v) was added to stop phosphohydrolysis. Free phosphate was determined by molybdivanadophosphate method according to AOAC (1984).

Isolation of phytase producing bacteria

Rumen fluid was sampled from the same sheep just before and 0.5, 1.0, 2.0, 3.0, 5.0 and 7.0 h after morning feeding. Sampled rumen fluid was squeezed through double layers of surgical gauze. Strained fluid was further subjected to ten-fold serial dilution with anaerobic dilution solution (Arakaki et al., 1994). The samplings were repeated three consecutive days. Phytase screening medium (PSM; Howson and Davis, 1983), M10 (Caldwell and Bryant, 1966) and RGCA (Bryant and Burkey, 1953) media were used with following modifications. Both anaerobic prereduced media, M10 and RGCA, were modified by a reduction of inorganic phosphate concentration to 1/10 of original level and a supplementation of calcium phytate (0.5%). PSM plates were cultured under both aerobic and anaerobic conditions. M10 and RGCA were cultured under anaerobic condition. Anaerobiosis was achieved under $N_2/CO_2/H_2$ (80/10/10) atmosphere in an anaerobic chamber (Coy laboratory, Ann Arbor, MI, USA) when cultured on plates or under oxygen-free CO_2 when cultured in roll-tubes. These plates and roll-tubes were incubated at 37°C for 7 days. The clear zone around a colony distinguished phytase-positive bacteria and a colony forming unit (cfu) of phytase- positive bacteria per unit (ml) of rumen fluid was determined. These phytase-positive bacteria were thereafter isolated and transferred to Toryptic-Soya medium (Difco laboratories, Detroit, MI, USA). Identification of the isolates was done in accordance with Bergey's Manual of Determinative Bacteriology (Holt et al., 1993) and partial 16S rDNA sequences. Briefly, the isolates were checked for their cell morphology and Gram staining. They were further subjected to the major physiological tests. Genomic DNA was purified from the isolates (Murray and Thompson, 1980) and the almost complete 16S rRNA gene of the respective strains was amplified by polymerase chain reaction (PCR) using rTaq DNA polymerase (Toyobo, Tokyo), the forward primer EUB-50F (5'-cgcggatccAACAC ATGCAAGTCAAGTCGCGAAC-3') and the reverse primer EUB 1405R (5'-cgctgcagACGGGCGGTGTGT ACAAG-3'). The primers were constructed according to Giovannoni et al. (1990) and Schmidt et al. (1991). Reaction mixture was composed of 100 ng of template DNA, 40 pmol of primers, and 10U DNA polymerase in 100 ml consisting 10 mM tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and each 50 mM dNTPs. PCR was performed for 60 s at 94°C at first, and each of 30 cycles of PCR consisted of incubation for 60 s at 94°C for denaturation, 60 s at 55°C for annealing, and 90 s at 72°C for elongation. Amplified products were digested by *Bam* HI and *Pst* I. These *Bam* HI-*Pst* I fragments were further digested by *Sac* II and *Eag* I or *Eag* I alone respectively for kpu 001 and kpu 002. Resultant fragments were ligated to the appropriate restriction sites of pBleuscript II SK(+) (Stratagene, La Jolla, CA, USA) by ligation High Kit (Toyobo, Tokyo). *Escherichia coli* XL1-Blue were transformed and transformants were selected by blue-white selection on LB plates containing ampicillin (100 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D- galactoside; 1 mg/plate) and IPTG (Isopropyl-β-D-thiogalactopyranoside; 2.38 mg/plate). At least three cloned plasmids for each fragment were sequenced. The sequencing reaction was performed with a Takara Cycle Sequencing Kit (Takara, Kyoto) using T7 labeled forward primer and T3 reverse primer. Sequencing was carried out on an auto sequencer DSQ-1000L (Shimadzu, Kyoto). Obtained sequence data were analyzed for homology with known bacterial 16S rDNA sequences by BlastNet and further multiply aligned using CLUSTAL W ver. 1.6 (Thompson et al., 1994) with a selection of proteobacteria and coryneform bacteria as reference sequences that were obtained from the nucleotide sequence libraries (GenBank, DDBJ and EMBL) and the Ribosomal Database Project (Maidak et al., 1999).

RESULTS AND DISCUSSION

Wheat bran phytin degradation in bacterial suspension did not differ from those determined in cell-free rumen fluid (table 1). In intact wheat bran 25 μmol of phytin was estimated to be contained in a unit (1 g) of bran. A substantial amount of phytin (15 μmol/g) disappeared during incubation irrespective of the presence of bacteria. When bran was autoclaved to inactivate endogenous phytase, autoclaving itself decreased phytin content (10 μmol/g) about half as

Table 1. Residual phytin contents (μmol/g) of wheat bran before and after *in vitro* rumen incubation as affected autoclaving

Intact wheat bran	
Before incubation (A)	24.6±4.13
After incubation in	
Bacterial suspension (B)	10.5±1.17
Cell-free rumen fluid (C)	8.2±1.11
Amount of hydrolyzed in 4 h in	
Bacterial suspension (A-B)	14.1
Cell-free rumen fluid (A-C)	16.4
Autoclaved wheat bran	
Before incubation (A')	10.4±0.73
After incubation in	
Bacterial suspension (B')	7.3±0.22
Cell-free rumen fluid (C')	4.7±0.13
Amount of hydrolyzed in 4 h in	
Bacterial suspension (A'-B')	3.1
Cell-free rumen fluid (A'-C')	5.7

Intact or autoclaved wheat bran were incubated in either cell-free rumen fluid or bacterial fraction at 37℃ for 4 h. After washing in citrate buffer, residual bran was treated with crude wheat phytase (Sigma P1259) to release phosphorous. Resultant free phosphorus was considered to be related with residual amount of phytin in bran after incubation with cell-free rumen fluid or rumen bacterial fraction.

Amount of phosphorus was converted to the amount of phytic acid.

Values are means for 3 determinations with standard deviations.

much as intact bran. In this condition 4 to 5 μmol/g of phytin was phosphohydrolyzed during incubation, and again no difference was observed between incubations in bacterial suspension and in cell-free rumen fluid. The presence of free-phytase in the rumen may explain the phosphohydrolyzation of phytin in the cell-free rumen fluid. Although this method did not give the exact rate of phosphohydrolyzation of phytin in bran, it may still suggest the less important role of rumen bacteria in phytin phosphohydrolyzation. When we compared the degradation rate in intact bran with that of autoclaved bran, the former (62.5%) was relatively higher than the latter (40.0%). The difference (22.5%) may suggest the important role of endogenous phytase in phytin degradation in the rumen at least in the case of wheat bran. The bacterial phospho-hydrolyzing activity was suggested not to be high. This situation was further evidenced by the relative low activity of bacterial crude enzyme toward the sodium phytate. Figure 1 shows phosphohydrolysis of sodium phytate. The activity is expressed as mg phosphate released per unit of rumen fluid (100 ml). In other words, figure 1 indicates the relative importance for phytin phosphohydrolyzation of protozoa and bacteria living in a same volume (100 ml) of

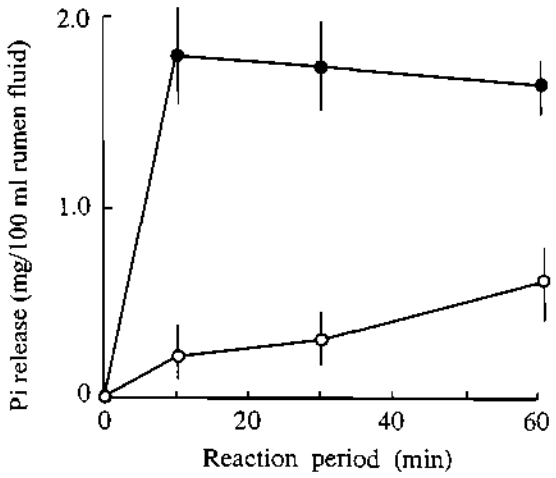

Figure 1. Pi release (mg) from Na-Phytate by protozoal and bacterial fraction each prepared from 100 ml rumen fluid. Na-Phytate was incubated in either crude enzyme preparations of protozoal (●) or bacterial (○) fractions. Bars represent standard deviations for 3 determinations.

rumen fluid. As shown in figure 1, phospho-hydrolyzing activity of protozoal population showed an approximately 7 times higher initial velocity than that of the bacterial population. Bacterial activity was, therefore, unexpectedly low. The less important role of rumen bacterial flora in phytin phosphohydrolyzation in the rumen was again supported by the fact that none of the phytase-positive bacteria was isolated under anaerobic condition irrespective of the medium used. Two distinct bacterial colonies showing clear zones were seen on PSM plates incubated under aerobic condition. One bacterium exhibited pinkish opaque colonies that made a larger halo, the other yellowish colonies making a relatively small halo. The numbers of these phytase-positive bacteria in the rumen increased from undetectable level to 1.7×10^5 cfu/ml 1.0 h after morning feeding, thereafter declined to the initial levels at 7.0 h after morning feeding (figure 2). These bacteria were isolated and designated as strains Kpu 001 and Kpu 002, respectively. Kpu 002 was twice as much as Kpu 001 at any sampling period. Kpu 001 was a Gram negative non-motile straight rod and able to grow both under anaerobic and aerobic condition. It exhibited the following characteristics; catalase test positive, oxidase test negative, indole test negative, sulfide production negative, methyl red test positive, Voges-Proskauer test negative, urease positive, growth on KCN positive, Simmons citrate reaction positive, lysine decarboxylase reaction positive and gelatin liquefaction negative. These physiological and morphological results suggested Kpu 001 to belong the genus *Klebsiella* and most likely to be *K. pneumoniae*. Kpu 002 was a Gram positive non-motile and

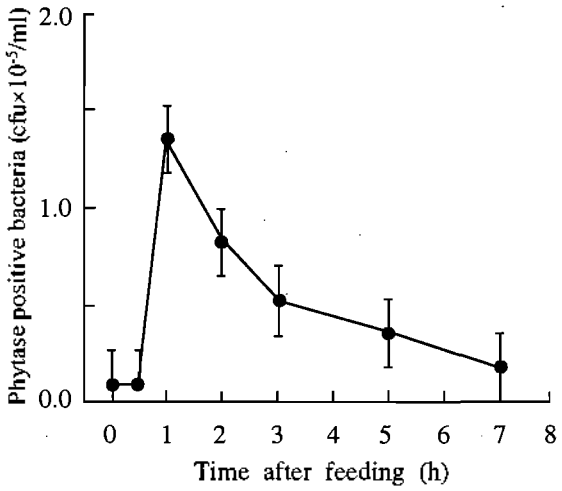

Figure 2. Number (cfu $\times 10^5$/ml) of ruminal phytase positive bacteria. Rumen fluid was sampled just before and 0.5, 1.0, 2.0, 3.0, 5.0, 7.0 after morning feeding and diluted to the appropriate concentration before applied on phytase screening medium. Details see text.

non-spore forming rod and able to grow under both anaerobic and aerobic conditions. It possessed catalase activity. The results suggested Kpu 002 belongs to the genus *Corynebacterium*. Phylogenetic analyses on 16S rDNA sequences indicated that Kpu 001 (Accession No.: AB012208) was closely related to *Klebsiella pneumoniae*, and Kpu 002 (Accession No.: AB012207) to *Corynebacterium bovis* or *C. vitarumen*. The population sizes of these facultative anaerobic bacteria were as low as 2×10^5 cfu/ ml indicating that these bacteria were not major constituents in the rumen. Their numbers were strongly affected by feeding: They might grow well under higher redox potential than observed in the rumen after feed ingestion. Since these bacteria did not show any clear haloes on either PSM plates or two Ca-phytate containing anaerobic media when grown under anaerobic condition, aerobic growth was essential for these facultative anaerobes to produce phytase. This observation agrees with those of Greiner et al. (1997) who indicated that *Klebsiella* sp. produced phytase only under aerobic condition.

CONCLUSION

Two facultative anaerobic bacteria, *Klebsiella* sp. and *Corynebacterium* sp., were isolated as phytase-producing organisms in the rumen. The population size of these bacteria indicated that these were relatively minor bacterial group in the rumen. The isolation of phytase-positive strict anaerobes was failed. Indeed, phytin phosphohydrolyzing activity of the rumen bacterial fraction was suggested to be lower than that of the protozoal fraction. Dietary phytase was indicated not to be ignored in phytin phosphohydrolysis in the rumen.

ACKNOWLEDGMENTS

This experiment was financially supported by Grant-in-Aid for Scientific Research from Ministry of Education, Science, Sport and Culture (No. 09660290). Special thanks are given to Professors T. Takeba and H. Yamashita, Laboratory of Applied Biology, Kyoto Prefectural University for their cooperation in the sequencing work. Valuable suggestions in phylogenetic works given by Dr. K. Tajima, STAFF Institute, Tsukuba, are also greatly acknowledged.

REFERENCES

AOAC. 1984. Official Methods of Analysis (14th Ed.) Association of Official Agricultural Chemists, Washington, DC. USA.

Arakaki, C., M. Mitsumori, H. Itabashi, S. Shirasaka and H. Minato. 1994. Influence of the presence of the presence of protozoa on the rumen microbial-population of cattle. J. Gen. Appl. Microbiol. 40:215-226.

Bryant, M. P. and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in thebovine rumen. J. Dairy Sci. 36:205-217.

Caldwell, D. R. and M. P. Bryant. 1966. Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. Appl. Microbiol. 14:794-801.

Cromwell, G. L., R. D. Coffey, H. J. Monegue and J. H. Randolph. 1995. Efficacy of low-activity, microbial phytase in improving the bioavailability of phosphorus in corn-soybean meal diets for pigs. J. Anim. Sci. 73:449-456.

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

Giovannoni, S. J., T. B. Britschgi, C. L. Moyer and K. G. Field. 1990. Genetic diversity in Sargasso sea bacterio-plankton. Nature. 345:60-63.

Greiner, R., E. Haller, U. Konietzny and K. D. Jany. 1997. Purification and characterization of a phytase from Klebsiella terigena. Arch. Biochem. Biophys. 341:201-206.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley and S. T. Williams. 1993. The Bergey's Manual of Determinative Bacteriology. 9th ed. Williams & Wilkins, Baltimore. p. 787.

Khan, N. 1996. Tackling the phosphate burden. Feed Mix. 4:22-26.

Maidak B. L., J. R. Cole, C. T. Parker Jr, G. M. Garrity, N. Larsen, B. Li, T. G. Lilburn, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje and C. R. Woese. 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. 27:171-173.

Murray, M. G. and W. F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4325.

Reid, R. L. and M. C. Franklin. 1947. The utilization of phytate phosphorus by sheep. Aust. Vet. J. 23:136-140.

Schmidt, T. M., E. F. Delong and N. R. Pace. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. 173:4371-4378.

Simons, P. C. M. and H. A. J. Versteegh. 1990. Improvement of phosphorous availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64:525-540.

Thompson, J. D., D. G. Higgins and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.