Microbiology (1998), **144**, 1565–1573 Printed in Great Britain

Phytase activity of anaerobic ruminal bacteria

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Phytase catalyses the release of phosphate from phytate (*myo*-inositol hexakisphosphate), the predominant form of phosphorus in cereal grains, oilseeds and legumes. The presence of phytase activity was investigated in 334 strains of 22 species of obligately anaerobic ruminal bacteria. Measurable activities were demonstrated in strains of *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella ruminicola*, *Mitsuokella multiacidus* and *Treponema* spp. Strains isolated from fermentations with cereal grains proved to have high activity, and activity was particularly prevalent in *S. ruminantium*, with over 96% of the tested strains being positive. The measured phytase activity was found exclusively associated with the bacterial cells and was produced in the presence of approximately 14 mM phosphate. The most highly active strains were all *S. ruminantium*, with the exception of the one *Mitsuokella multiacidus* strain examined. Phytase activity varied greatly among positive strains but activities as high as 703 nmol phosphate released (ml culture)$^{-1}$ were measured for a *S. ruminantium* strain and 387 nmol phosphate released (ml culture)$^{-1}$ for the *Mitsuokella multiacidus* strain.

Keywords: phytase, *Selenomonas*, phosphate, anaerobic, rumen

INTRODUCTION

Phytate (*myo*-inositol hexakisphosphate) is the predominant form of phosphorus in cereal grains, oilseeds and legumes (Reddy *et al.*, 1982; Al-Asheh & Duvnjak, 1995). However, monogastric animals, such as pigs, poultry and fish, utilize this source of phosphate poorly at best, lacking the requisite gastrointestinal tract enzyme(s) for release of the phosphate from the organic complex of phytate (Common, 1989). In soil and water environments the catalysed release of the phosphate occurs; hence phytate in manure can pose a serious phosphorus pollution problem contributing to the eutrophication of surface waters in areas of the world where monogastric livestock production is intensive (Pen *et al.*, 1993; Volfová *et al.*, 1994). In addition, producers have to use expensive supplementary feed phosphorus to meet animals' dietary requirements, even though this phytate phosphate is unutilized. Further, phytate has anti-nutritive properties including formation of complexes with protein and divalent cations (Al-Asheh & Duvnjak, 1995). Phytases have been investigated as a possible solution to these problems, and have been reported not only to increase phosphate utilization efficiency from phytate in feeds but also to decrease phosphorus pollution (Pen *et al.*, 1993; Broz *et al.*, 1994).

Industrial production of phytase currently utilizes the soil fungus *Aspergillus*, on which a considerable amount of research has been conducted (Ullah, 1988a, b; Van Hartingsveldt *et al.*, 1993). Phytases have also been characterized from other aerobic fungi (Segueilha *et al.*, 1993; Mitchell *et al.*, 1997), plants (Gibson & Ullah, 1988; Hübel & Beck, 1996), animals (Maga, 1982), bacteria (Shimizu, 1992; Greiner *et al.*, 1993; Yoon *et al.*, 1996) and *Paramecium* (Van der Kaay & Van Haastert, 1995). However, one group of micro-organisms that has not been examined are the strict anaerobes, in particular ruminal anaerobes, which are known to have highly active enzymes involved in feed degradation (Teunissen & Op den Camp, 1993).

Ruminants, in contrast to monogastrics, possess digestive phytase activity and thus are potentially able to utilize phytate, rendering phosphate supplementation

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unnecessary on high-grain diets. Digestion of cereal grains, with their starch granules in a protein matrix and associated phytate (Reddy *et al.*, 1982; McAllister *et al.*, 1993), is carried out largely by the ruminal bacterial and protozoal populations (Bonhomme, 1990; McAllister *et al.*, 1994). While amylases have been cloned from *Streptococcus bovis* (Satoh *et al.*, 1993), and numerous bacterial species have been shown to be proteolytic (Wallace & Cotta, 1988), to our knowledge only a single report has attributed phytase activity to ruminal microorganisms (Raun *et al.*, 1956). However, the microbial suspensions used were prepared by centrifugal sedimentation (Cheng *et al.*, 1955) and were probably contaminated with microscopic particles of plant material; since plants produce phytases themselves the study was inconclusive as to the origin of the phytase activity. Thus, although the study of Raun *et al.* (1956) raises the possibility that ruminal phytase production may be microbial in nature this possibility has not been explored and the unique capacity of ruminants to utilize phytate has largely been ignored. To explore and define this ability of the rumen microflora a survey of phytase activity among pure cultures of predominant rumen bacteria was undertaken.

METHODS

Ruminal samples. Ruminal samples were obtained from three cannulated Hereford steers each on a different diet: 90% barley grain/10% hay, 55% barley grain/45% hay, and 100% hay. The steers were pre-adapted for one month on these diets prior to rumen fluid collection and were sampled on three replicate days. Rolled barley was used as the cereal grain source and hay cubes (70% alfalfa hay and 30% barley straw) were used as the roughage source. Collected rumen fluid samples were strained through six layers of cheesecloth and then the samples were centrifuged according to McAllister *et al.* (1992) to separate them into supernatant, bacterial and particulate (protozoal/feed particle/fungal) fractions. Pelleted fractions were washed once with 0·1 M sodium acetate buffer (pH 5·0) and resuspended to original volume in the same buffer. Total free phosphate was measured in the supernatant fraction and phytase activity was measured in all fractions.

Bacterial strains, media and growth conditions. The 334 strains of bacteria used in this survey are listed in Table 1, along with the ruminal origin and source or reference of the strain. The Rusitec and *in vitro* incubations from which the isolations were made both used cereal grains as the fermentable carbohydrate source and were inoculated with rumen fluid from two steers fed a diet of 50% wheat, 35% alfalfa and 15% barley straw. Modified Scott and Dehority medium (Scott & Dehority, 1965) supplemented with 5% (v/v) clarified rumen fluid, 0·3% (w/v) glucose, 0·3% (w/v) cellobiose and 0·45% (w/v) soluble starch was used for growth of the bacteria. This medium has a phosphate concentration of approximately 14 mM. All cultures were grown overnight at 39 °C prior to experimental inoculation of 100 µl into tubes with 5 ml fresh medium and again grown overnight before sampling for phytase activity. For the phytase plate-screening, agar plates (1·8%, w/v, agar) of the modified Scott and Dehority medium, with 2% (w/v) sodium phytate (phytic acid, dodecasodium salt from corn, Sigma P8810) added, were similarly streaked from overnight cultures and incubated for 120 h at 39 °C in an anaerobic chamber with an atmosphere of 90% CO_2/10% H_2. Strong demonstration of zones of clearing required this length of growth even though good growth was normally visible by 24 h. For determination of phytase activity, on agar plates and in liquid, all cultures were grown in duplicate and replicated on at least two separate days. The anaerobic technique of Hungate (1950) as modified by Bryant & Burkey (1953) was used throughout for bacterial growth.

Sample preparation. Bacterial cultures were prepared for assaying by centrifugation of 1·5 ml culture at 18000 *g*. The cell-free supernatant was removed and the bacterial pellet was resuspended in 0·1 M sodium acetate buffer (pH 5·0) and recentrifuged. The resulting wash supernatant was removed and the pellet resuspended in 750 µl buffer (a twofold concentration of original culture). Appropriate dilutions of the samples, if necessary, were also done with the 0·1 M sodium acetate buffer (pH 5·0), with the activities corrected so as to be reported per ml of original culture.

Phytase assays. Phytase plate-screening was carried out aerobically by washing the colonies from the agar surface and flooding the Petri plates with a 2% (w/v) cobalt chloride solution. After 5 min incubation at room temperature the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of a 6·25% (w/v) ammonium molybdate solution and a 0·42% (w/v) ammonium vanadate solution. Following a further 5 min incubation the solution was removed and the plates were examined for zones of clearing indicative of phytase activity, without interference from clearing zones caused by acid production (unpublished results).

For the quantitative phytase assay, 150 µl sample was added to 600 µl 0·2% (w/v) sodium phytate prepared daily in 0·1 M sodium acetate buffer (pH 5·0). The reaction was incubated for 30 min at 39 °C before being stopped by the addition of 750 µl 5% (w/v) trichloroacetic acid, followed by 750 µl phosphomolybdate colour reagent. The colour reagent was freshly prepared by the addition of 4 vols 1·5% (w/v) ammonium molybdate solution in 5·5% (v/v) sulfuric acid to 1 vol. 2·7% (w/v) ferrous sulfate solution. Following a 5 min colour-development period, the liberated inorganic phosphate was measured as A_{700} with a Gilford 300-N spectrophotometer. One unit (U) of phytase was defined as the amount of enzyme required to liberate 1 nmol P_i min^{-1} under the given assay conditions, and is given per ml of original culture, with the standard error (SE). Duplicate samples were taken from duplicate tubes and replicated on at least two separate days.

RESULTS

Ruminal phytase activity

The ability of the rumen environment to hydrolyse phytate was demonstrated by the measured phytase activities of different ruminal fractions under different dietary conditions (Fig. 1). More activity was apparent on the higher barley grain diets than with hay alone, with the total activity split between the supernatant and bacterial fractions. The effect of the diet was particularly evident in the bacterial fraction. Total free phosphate levels on the diets were also measured. The 90% barley grain/10% hay diet demonstrated the highest levels, at $17{\cdot}5 \pm 2{\cdot}2$ mM ($\pm$ SE, $n = 3$), and the 100% hay diet the lowest levels, at $4{\cdot}3 \pm 0{\cdot}1$ mM. The 55% barley

Table 1. Bacterial strains used and source of isolation

Species	Strain(s)	Origin*	Source†
Anaerovibrio lipolytica	5S	Ovine	Rowett
	YB64	Bison	LRC
Bacillus sp.	YB44, YB62	Bison	LRC
	YM73	Moose	LRC
	YL118	Elk	LRC
	YT41	*in vitro*	LRC
Butyrivibrio fibrisolvens	UC142	NK	Rowett
	E14, B49	Svalbard reindeer	Orpin
	49	Bovine	Bryant
	H17C	Bovine	Whitehead
	J8-7, H4-3	Bovine	LRC
	YB8, YB10, YB11, YB16, YB21s, YB28, YB30, YB32, YB35, YB45	Bison	LRC
	YM70, YM80, YM87	Moose	LRC
	YL13, YL15, YL16, YL17, YL19, YL21, YL25, YL30, YL38, YL99, YL107, YL110, YL133, YL139, YL256A	Elk	LRC
	YT10, YT107, YT133	*in vitro*	LRC
	YR2B, YR42, YR76, YR109, YR121, YR127	Rusitec	LRC
Clostridium sp.	PDO 4822	Bovine	Bryant
Coprococcus sp.	$Pe_1$3, $Pe_1$12, $Pe_1$5	Bovine	Jones
Enterococcus faecalis	YL1	Elk	LRC
	C11, H10, H55	Bovine	LRC
Eubacterium ruminantium	B_1C_{23}	Bovine	Bryant
	G1-1_1	Bovine	LRC
	YR39, YR43	Rusitec	LRC
Eubacterium sp.	YL111, YL116, YL142	Elk	LRC
Fibrobacter succinogenes	S85	Bovine	Bryant
	135, WB7	Water buffalo	Kudo
	N22-8, L14-31	Bovine	LRC
	YL4, YL134, YL163b	Elk	LRC
Lachnospira multipara	D32, 40	Bovine	Bryant
	L14-8	Bovine	LRC
	YR101	Rusitec	LRC
Lactobacillus ruminis	27780	Bovine	ATCC
Lactobacillus sp.	LB17	Ovine	Rowett
	GA1	Bovine	Bryant
	L12-4	Bovine	LRC
	YR1, YR8, YR9, YR12, YR17, YR22, YR27, YR28, YR31, YR57, YR62, YR87, YR88, YR92, YR102, YR108	Rusitec	LRC
Megasphaera elsdenii	B159, T81	Bovine	Bryant
	LC1, AAW106, J1	Ovine	Rowett
	YT91, YT111, YT153	*in vitro*	LRC
	YR10, YR38, YR60, YR78, YR99, YR114	Rusitec	LRC
Mitsuokella multiacidus	46/5(2)	Ovine	Rowett
Peptostreptococcus sp.	B43	Bovine	Bryant
Prevotella ruminicola	GA33, 23, $B_1$4	Bovine	Bryant
	S-pec-2, 223/M2/7	Ovine	Rowett
	19188	Bovine	ATCC

Table 1 *continued overleaf*

Table 1 *(cont.)*

Species	Strain(s)	Origin*	Source†
Prevotella ruminicola (cont.)	YB2, YB5, YB7, YB17, YB18, YB29, YB38, YB46, YB48, YB56b, YB73	Bison	LRC
	YM14b, YM146	Moose	LRC
	YL18, YL97, YL104, YL106, YL114, YL189, YL235, YL237, YL253, YL254	Elk	LRC
	YT3, YT8, YT67, YT72, YT77, YT85, YT93, YT106, YT108	*in vitro*	LRC
	YR2a, YR6, YR11, YR34, YR68, YR74, YR96, YR103, YR111, YR128	Rusitec	LRC
Ruminobacter amylophilus	WP91, WP109, WP225	NK	Rowett
	YT103	*in vitro*	LRC
Ruminococcus albus	C1	Svalbard reindeer	Orpin
	ND4, ND7, ND8, ND9, ND10-1	Malaysian deer	Kudo
Ruminococcus flavefaciens	$B_1$46	Bovine	Bryant
	136	Bovine	LRC
	YB65, YB72, YB74	Bison	LRC
	ND6	Malaysian deer	Kudo
	NWB6	Water buffalo	Kudo
	NMD7, NMD8, NMD9	Lesser mouse deer	Kudo
	NLMD1	Larger mouse deer	Kudo
Selenomonas ruminantium	D, HD4, GA192	Bovine	Bryant
	S-pec-1, WPL/151/1	NK	Rowett
	19205	Bovine	ATCC
	YB1, YB4dot, YB4, YB6, YB14, YB15, YB31, YB42, YB49b, YB52, YB55s, YB56a, YB57	Bison	LRC
	YM59, YM63, YM69, YM93	Moose	LRC
	YL23, YL24, YL26, YL28, YL29, YL33, YL40clr, YL44, YL100, YL123, YL127, YL143	Elk	LRC
	JY35, YT6, YT7, YT14, YT16, YT18, YT27, YT29, YT30, YT39, YT40, YT43, YT50, YT53, YT56, YT86, YT92, YT110, YT114, YT118, YT131, YT135, YT141	*in vitro*	LRC
	YR21, YR47, YR89, YR105, YR106	Rusitec	LRC
Staphylococcus sp.	1592/14 Staph 2, V105, 1592/14 Staph 1	Bovine	LRC
	YM62	Moose	LRC
	YL121, YL163w	Elk	LRC
Streptococcus bovis	45 S1	NK	Rowett
	9809	NK	ATCC
	YB54, YB75, YB78	Bison	LRC
	YM78w, YM88, YM151w, YM82	Moose	LRC
	YL3, YL7a, YL98	Elk	LRC
	YT21, YT33, YT38, YT46, YT61, YT78, YT79, YT87, YT100, YT104, YT119, YT126, YT127, YT136, YT137, YT152, YT155, YT156, YT168, YT174, YT175, YT180, YT185, YT200, YT201, YT205, YT206, YT223	*in vitro*	LRC
	YR5, YR49, YR71, YR84, YR97, YR115	Rusitec	LRC
	NM1, NM2, NM7	Lesser mouse deer	Kudo

Table 1 *(cont.)*

Species	Strain(s)	Origin*	Source†
Succinovibrio dextrinisolvens	24	Bovine	Bryant
	P3	Bovine	LRC
	YB12, YB26	Bison	LRC
	YM61, YM79, YM86, YM90	Moose	LRC
	YL8, YL103, YL137, YL219	Elk	LRC
Treponema bryantii	$B_2$5	Bovine	Bryant
Treponema sp.	YB3, YB13, YB20, YB21, YB23, YB23b, YB33, YB41, YB50, YB77	Bison	LRC
	YL49	Elk	LRC
	DT1	Malaysian deer	Kudo
Unidentified strains	YB61	Bison	LRC
	YT42	*in vitro*	LRC
	YR14, YR19, YR23, YR37, YR66, YR100	Rusitec	LRC

* Origin of sample from which strain was isolated: *in vitro*, an end-point fermentation with cereal grains; Rusitec, a continuous fermentation (Czerkawski & Breckenridge, 1977) with cereal grains; NK, of uncertain origin.

† Rowett, Rowett Research Institute (Aberdeen, UK); LRC, Agriculture and Agri-food Canada Research Centre (Lethbridge); Orpin, C. G. Orpin (CSIRO, Australia); Bryant, M. P. Bryant (University of Illinois, Urbana); Whitehead, T. R. Whitehead (USDA, Peoria, IL, USA); Jones, G. A. Jones (University of Saskatchewan, Saskatoon, Canada); Kudo, H. Kudo (NIAH, Japan); ATCC, American Type Culture Centre (Rockville, MD, USA).

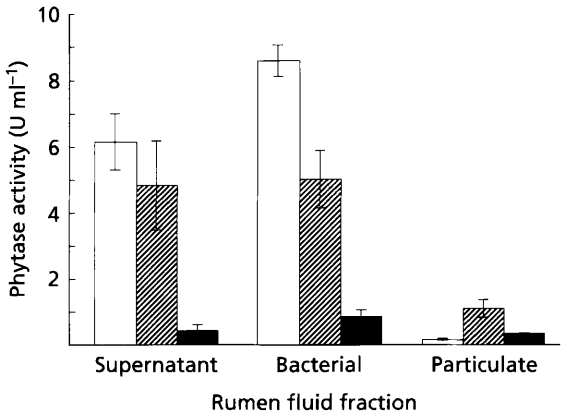

Fig. 1. Phytase activity in fractionated rumen fluid samples from steers fed different diets: □, 90% barley grain/10% hay; ▨, 55% barley grain/45% hay; ■, 100% hay. Bars indicate SE ($n = 3$).

grain/45% hay diet gave intermediate levels: 8·1 ± 4·3 mM.

Plate-screening for phytase activity

Initial qualitative screening of 101 of the pure rumen bacteria cultures indicated phytase activity, as determined by clearing zones on phytate-containing plates, in strains of *Prevotella ruminicola*, *Ruminobacter amylophilus*, *Selenomonas ruminantium* and *Streptococcus bovis*, with virtually all the *Selenomonas ruminantium* strains having activity (Table 2). These clearing zones ranged in size from the diameter of the associated colony to *Selenomonas ruminantium* zones of clearing several-fold larger than the colony. All *Prevotella ruminicola*, *Ruminobacter amylophilus* and *Streptococcus bovis* zones of clearing were of about the same size as the colonies with which they were associated. None of the other species tested demonstrated any clearing zones and there did not appear to be any influence of the original source of the isolate (data not shown).

Table 2. Plate-screening of pure bacterial cultures for phytase activity

Species	Positive strains	Negative strains
Bacillus sp.	0	1
Butyrivibrio fibrisolvens	0	16
Enterococcus faecalis	0	1
Eubacterium sp.	0	3
Fibrobacter succinogenes	0	4
Lactobacillus sp.	0	4
Prevotella ruminicola	3	7
Ruminobacter amylophilus	1	0
Ruminococcus albus	0	2
Ruminococcus flavefaciens	0	4
Selenomonas ruminantium	16	1
Streptococcus bovis	15	20
Succinovibrio dextrinisolvens	0	3
Totals	**35**	**66**

Table 3. Phytase activity of rumen bacteria

Species	No. of strains				
	Phytase activity (U ml^{-1}):				
	>125	25–125	5–25	<5	None
Anaerovibrio lipolytica	0	0	0	0	2
Bacillus sp.	0	0	0	1	4
Butyrivibrio fibrisolvens	0	0	0	0	44
Clostridium sp.	0	0	0	0	1
Coprococcus sp.	0	0	0	0	3
Enterococcus sp.	0	0	0	0	4
Eubacterium sp.	0	0	0	0	7
Fibrobacter succinogenes	0	0	0	0	8
Lachnospira multipara	0	0	0	0	4
Lactobacillus sp.	0	0	0	0	20
Megasphaera elsdenii	0	0	5	2	7
Mitsuokella multiacidus	1	0	0	0	0
Peptostreptococcus sp.	0	0	0	0	1
Prevotella ruminicola	0	5	2	3	38
Ruminobacter amylophilus	0	0	0	0	4
Ruminococcus albus	0	0	0	0	6
Ruminococcus flavefaciens	0	0	0	0	11
Selenomonas ruminantium	8	16	18	19	2
Staphylococcus sp.	0	0	0	0	6
Streptococcus bovis	0	0	0	0	49
Succinovibrio dextrinisolvens	0	0	0	0	12
Treponema sp.	0	0	0	0	11
Unidentified	0	0	0	0	8
Totals	**9**	**21**	**25**	**27**	**252**

Quantitative assay of phytase activity

Having determined the presence of apparent phytase activity in some of the pure strains it was decided to quantify this activity and to correlate the appearance of a clearing zone with actual enzymic release of phosphate from phytate, as well as to screen further strains for phytase activity. Quantitatively, it was seen that the overwhelming number of positive strains, and particularly strongly positive strains, were *Selenomonas ruminantium*, with a substantial percentage of *Megasphaera elsdenii* isolates also being positive but with lower activities (Table 3). The *Selenomonas ruminantium* strains with the highest activities (±SE, $n = 4$) were JY35 (703·6±14·0 U ml^{-1}), YT118 (529·5±6·9 U ml^{-1}), YT131 (502·0±6·5 U ml^{-1}), YT141 (393·7±41·5 U ml^{-1}), YT86 (312·8±14·1 U ml^{-1}), YR106 (240·5±6·7 U ml^{-1}), YT135 (235·4±28·1 U ml^{-1}) and YR47 (199·1±8·1 U ml^{-1}). Other strains from the same sources as these, and from other sources, had lower levels of activity all the way down to strain YB15 with an activity of 0·49±0·17 U ml^{-1}. Strain D (75·06±5·87 U ml^{-1}) was the only one from another institution to evidence more than 25 U ml^{-1} activity. The *Megasphaera elsdenii* strains had activities from 13·99±0·12 U ml^{-1} (strain YR99) to 3·30±0·12 U ml^{-1} (strain YR78). The only *Mitsuokella multiacidus* strain had a high level of activity at 387·5±34·8 U ml^{-1}. *Prevotella ruminicola* also had a noticeable proportion of phytase positive strains. Strains YL106, YL189, YL18 and YL97 had similar activities at 47·18±0·99, 41·66±1·06, 40·91±3·29 and 38·58±0·98 U ml^{-1} respectively. Strain S-pec-2 had an activity of 27·65±1·21 U ml^{-1} and strains GA33 and 23 had roughly fivefold lower levels of activity. Strains $B_1$4, YT106 and YT77 had only minimal activities. A *Bacillus* strain (YB62, 3·53±0·12 U ml^{-1}) and two *Treponema* strains ($B_2$5 and YB41, 0·88±0·43 and 0·49±0·07 U ml^{-1} respectively) were the only other positives. Despite a positive result in the plate-screening, none of the *Streptococcus bovis* or *Ruminobacter amylophilus* strains demonstrated any phytase activity.

In all cases the phytase activity was associated with the cell pellet, and there was no activity in the cell-free or wash supernatant of any of the isolates, positive or negative (data not shown). Further, there was no requirement for induction of the measured activity by the addition of phytate and the activity was produced in the presence of approximately 14 mM phosphate in the growth media used.

Although there was substantial variation in the phytase activity of strains of the same species, all the bacterial strains grew well and this variation was not a reflection

of different levels of growth which, while variable, did not appear to correlate with the level of phytase activity (data not shown). Many of the strongly positive *Selenomonas ruminantium* strains and all the *Megasphaera elsdenii* strains were isolated at LRC from *in vitro* or continuous fermentation (Rusitec) incubations with cereal grains, but such conditions did not always result in high-activity strains, and strains with good activity were isolated from other sources (Table 1). The only two negative *Selenomonas ruminantium* strains, YL44 and YL28, were isolated from elk. The phytase-positive *Prevotella ruminicola* strains were mostly isolated from elk, with few positive strains isolated from *in vitro* or continuous fermentation sources even though numerous *Prevotella* strains were isolated from these same sources that contained the highly active *Selenomonas* strains. The highly active *Mitsuokella multiacidus* was isolated at the Rowett Research Institute.

DISCUSSION

Phytase supplementation to poultry and swine diets is an area of growing interest as it has the potential both to improve feed efficiency and growth rate of the animals and to decrease environmental pollution (Pen *et al.*, 1993; Broz *et al.*, 1994). In contrast, the other major livestock group, ruminants, requires less phosphate supplementation, possibly in part because of higher levels of phytase activity in these animals. In our study, rumen phytase activity appeared to be largely of bacterial origin, as had been previously demonstrated (Raun *et al.*, 1956), and the activity increased with higher grain diets. Such an increase in phytase activity may well have been in response to the higher levels of phytate in cereal grains as compared to forages (Reddy *et al.*, 1982). Relatively little activity was apparent in the particulate (protozoal/feed particle/fungal) fraction of the ruminal fluid, and screening of pure cultures firmly established the presence of phytase activity within the ruminal bacterial flora.

Initial plate-screening indicated phytase activity among certain of the genera tested, but when quantitative measurements of phytase activity were made some discrepancies with the plate-screening results were found. While *Selenomonas ruminantium* and to a lesser extent *Prevotella ruminicola* strains demonstrated phytase activity, including all positive plate cultures, none of the *Streptococcus bovis* or *Ruminobacter amylophilus* strains had any measurable activity, even though some of these strains had been positive in the plate-screening. This may possibly be explained as very low levels of activity observable only after prolonged incubation, as on the plates, or that activity may have been induced by the plate growth conditions, most notably the presence of phytate, as compared to the growth conditions for the quantitative assays. The zones of clearing for *Selenomonas ruminantium* that were several-fold larger than the colony may have been the result of extracellular enzymes or the release of enzymes from lysed cells, a consequence of the long incubation period necessary. There did not appear to be any false negatives from the initial plate-screening and it seems likely that this method is more sensitive than the quantitative phytase assay used in this experiment.

Comprehensive screening of rumen bacteria for phytase activity clearly showed a high level of occurrence among virtually all *Selenomonas ruminantium* strains irrespective of their source. There was, however, considerable variation in the actual activities measured, with the most active strains isolated from *in vitro* or continuous fermentations with cereal grains. This result is a good reflection both of the increased phytase active *in situ* on high-grain diets and of the increased *Selenomonas* population on such diets (Ricke *et al.*, 1996). These results also closely correspond to those for the *Megasphaera elsdenii* strains, although these strains showed considerably less activity. Further, it is interesting that the genera *Streptococcus* and *Ruminobacter*, both of which were positive in the initial plate-screening, are also associated with high-grain diets and starch digestion (Stewart & Bryant, 1988).

While some phytase activity was found in strains of *Bacillus*, *Treponema* and *Prevotella*, notably in strains isolated from elk, the highest levels of activity were found in the *Selenomonas ruminantium* strains, with the exception of the only *Mitsuokella multiacidus* strain examined. Under the growth conditions employed this activity was not induced and was found exclusively associated with cells; no activity was ever measurable in the supernatant. This fact raises a question as to the origin of the supernatant activity measured in the rumen (Fig. 1). Whether this activity is from another, possibly plant or animal, source or whether it is from an as yet unidentified rumen micro-organism remains unknown. There is also the possibility that this activity is the result of cell lysis or unexplored cell-regulated export within the rumen.

Both the production and regulation of the measured phytase activity in this study contrast with phytases from other sources, where the activity is under tight regulatory inhibition by phosphate levels and is exported extracellularly, as in *Aspergillus* and *Bacillus* (Volfová *et al.*, 1994; Shimizu, 1992), or is found periplasmically, as in *Escherichia coli* (Greiner *et al.*, 1993). While the free phosphate levels in the medium in our study were high, they were comparable to levels found in the rumen in this study and elsewhere (Durand & Kawashima, 1980). As such, inhibition of phytase activity by phosphate appears unlikely in ruminal bacteria because of the high levels of free phosphate normally encountered in the rumen. However, this does raise a question about the physiological function of the phytase activity in these bacteria if phosphate limitation is not a problem.

Although inherent differences in experimental conditions and purity of enzyme preparations make comparisons difficult, the initial crude levels of activity found in the ruminal bacteria compare favourably with those reported for *Aspergillus ficuum* (600 U ml^{-1}; Shimizu, 1993), *Bacillus subtilis* (44 U ml^{-1}; Shimizu, 1992) and *E. coli* (5600 U ml^{-1}; Greiner *et al.*, 1993). The

measured levels of phytase activity suggest that ruminal bacteria are a potential source of a highly active phytase that could be developed commercially. Ongoing studies are being conducted to further characterize the phytase activity of the ruminal strains and its regulation, if any, as well as to isolate bacteria with higher phytase activity from the rumen.

ACKNOWLEDGEMENTS

The authors would like to thank Drs G. D. Inglis and X. Lin for their constructive comments on the manuscript, and all the researchers and institutions noted in Table 1 for the distribution of the bacterial strains. This project was partially funded by Pioneer Hi-Bred International, Inc., and Alberta Agriculture Research Institute Matching Grant no. 95M814.

This paper is LRC contribution no. 3879748.

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Received 14 October 1997; revised 19 January 1998; accepted 24 February 1998.