J Sci Food Agric 82:398–404 (online: 2002)
DOI: 10.1002/jsfa.1047

Inhibition of amylases present in ruminal particle-associated micro-organisms

TF Martínez,* M Díaz and FJ Moyano
Departamento de Biología Aplicada, Área de Biología Animal, CITE II-B, Universidad de Almería, Campus Universitario de La Cañada, E-04120 Almería, Spain

Abstract: The effect of different substances potentially inhibitory of ruminal amylase activity in sheep was assessed using biochemical and electrophoretic assays. Most amylase activity was detected in the particle-associated fraction (70%) of the ruminal contents (which was selected for the assays) in comparison with the bacterial (21%) and extracellular (9%) fractions. Salts of divalent ions such as Sn^{2+}, Hg^{2+}, Cu^{2+} and Zn^{2+} produced 90, 82, 65 and 44% inhibition of amylase activity respectively when assayed at a relative concentration of 5×10^{-3} mol l^{-1}. Organic acids such as tannic, formic, ascorbic and benzoic acid produced 79, 48, 43 and 37% inhibition respectively, whereas chelators such as EDTA, EGTA and 1,10-phenanthroline produced an inhibition ranging from 32 to 37%. Substrate SDS-PAGE zymograms allowed the identification of different amylase-active bands in ruminal extracts, showing a wide range of relative molecular masses (from 36 to more than 100 kDa). Such zymograms also confirmed the effect of some inhibitors. The reversibility of the inhibitory effect of some of the assayed substances was assessed. $ZnSO_4$ was the most persistent inhibitory substance even at low concentrations and, owing to its low toxicity, appears to be an adequate substance to reduce the high *in vitro* ruminal degradation of starch. Implications for the process of enzymatic digestion of starch are discussed.

Keywords: amylase inhibition; amylase re-naturalisation; SDS-PAGE zymograms; starch hydrolysis

INTRODUCTION

Microbial fermentations taking place in the rumen allow ruminants to utilise feed resources otherwise inadequate for their nutrition. This feature undoubtedly represents an advantage under most of the conditions and production systems utilised for these animals. However, under intensive production systems where energy requirements are increased, high dietary levels of cereal grains are needed. It is recognised that ruminal micro-organisms utilise dietary starch extensively,[1] the rumen being the major site of its hydrolysis in the digestive tract,[2] with some exceptions such as corn and sorghum starch.[1,3] The ability to utilise starch as a carbon source is widespread among the different groups of bacteria, protozoa and fungi constituting the rumen microbial population,[4] although the amylolytic activity of protozoa and fungi seems to represent only a fraction of the whole starch degradation in the rumen.[5] However, the rumen protozoa also participate in the degradation of plant polysaccharides.[6,7]

Despite the importance of starch in diets for highly productive ruminants, the enzymatic mechanisms of its degradation by rumen micro-organisms have been less extensively studied when compared with other enzymatic activities. Moreover, most of such studies are mainly focused on extracellular amylase activity, which represents only a fraction of the total amylolytic activity present in the whole rumen.[8] On the other hand, it is widely recognised that the efficiency of intestinal digestion of starch (yielding glucose as the final product) is largely higher than its fermentation in the rumen, in which starch is converted to volatile fatty acids.[9] In addition, the excessive utilisation of cereal grains as a source of energy to meet the requirements of highly productive ruminants is one of the main causes of acidosis.[10] Some attempts have been made to reduce the extensive hydrolysis of readily fermentable starches (eg those contained in grains such as barley, wheat and oats), such as the use of formaldehyde[11] or micronisation,[12] in order to obtain less degradable substrates.

More detailed knowledge about enzymes involved in the utilisation of soluble carbohydrates in the rumen should allow the manipulation of carbohydrate hydrolysis, which may result in an optimised utilisation of the dietary energy by the ruminant, as well as preventing the associated metabolic disorders.[10,13,14] Practical application of such knowledge could reduce ruminal digestibility of starch-containing feeds by

* Correspondence to: TF Martínez, Departamento de Biología Aplicada, Área de Biología Animal, CITE II-B, Universidad de Almería, Campus Universitario de La Cañada, E-04120 Almería, Spain
E-mail: tomas@ual.es
Contract/grant sponsor: Consejería de Educación (Junta de Andalucía)
(*Received 8 August 2001; revised version received 19 October 2001; accepted 29 October 2001*)

partial inhibition of amylase activity, and also result in increased post-ruminal availability of starch. However, inhibition of ruminal amylase activity remains virtually unexplored, and no literature related to this topic has been found.

In this study the effect of several potential inhibitors of rumen amylase activity was evaluated after a preliminary assessment of the optimal conditions for the assays. The persistence and reversibility of the inhibitory effect must be evaluated, taking into account that this is an important feature in the particular environment of the rumen ecosystem, owing to the possibility of dilution of the substances acting as inhibitors. A novel method of preparing SDS-PAGE zymograms[15] was used to illustrate both the variability existing in ruminal amylase activities and their sensitivity to different inhibitors.

EXPERIMENTAL

Reagents

Reagents used were: ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetate (EGTA), *N*,*N*′-methylene-bis-acrylamide, Coomassie Blue Staining (BBC R-250), α-amylase inhibitor from *Phaseolus vulgaris*, and molecular mass protein standard (MWM) for electrophoresis (obtained from Sigma Chemical Co, St Louis, MO, USA); *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED) and ammonium persulphate (obtained from Pharmacia Biotech, Uppsala, Sweden); α-amylase inhibitor from *Triticum aestivum* protein (obtained from ICN Biomedicals Inc, Irvine, CA, USA); and acrylamide (from Bio-Rad, Richmond, CA, USA). All other reagents were purchased in the purest form available from different commercial sources.

Animals

Four adult, cannulated, non-lactating females of Segureña sheep (local breed) were used. The animals were fed twice daily with a diet based on dehydrated alfalfa and milled corn grain in a 3:1 DM ratio, vitamin and mineral supplement and free access to water. EU requirements related to laboratory animal welfare were met.

Extraction of ruminal fluid and preparation of semi-purified extracts

Ruminal fluid was obtained by means of a silicone tube (13 mm internal diameter) attached to a vacuum source through each ruminal cannula 4 h after the morning feeding. Pooled ruminal fluid obtained from different animals was used as the enzyme source to avoid variations in enzyme activity from individual samples. After extraction the ruminal fluid was placed in preheated (39 °C) Dewar vessels gassed with CO_2. The ruminal fluid was not strained but was centrifuged within 30 min of collection (10 min, $500 \times g$, 4 °C) in 10 ml capped tubes previously purged with CO_2. The pellet containing the particulate fraction with associated microbes (fraction 1) was separated from the supernatant, and the latter was then re-centrifuged ($20\,000 \times g$, 20 min, 4 °C) in 10 ml capped tubes purged with CO_2 in order to separate an extracellular fraction (fraction 2, supernatant) and a bacterial fraction (fraction 3, pellet). In order to liberate enzymes from microbial cells, both particulate (fraction 1) and bacterial (fraction 2) fractions were resuspended in distilled water to their original volume (10 ml per tube) and then disrupted by sonication (Microson XL2000, Misonix Inc, Farmingdale, NY, USA; fitted with a 3.25 mm sonotrode; 12 W output power in 10 periods of 12 s duration each). Samples were kept on ice during the process and then centrifuged ($20\,000 \times g$, 20 min, 4 °C). Supernatants were stored at −20 °C and used as enzyme source in the assays. Assays were carried out using particulate fraction enzymatic extracts unless stated otherwise.

Amylase activity assays

Total amylase activity in ruminal extracts was measured according to the procedure described by Robyt and Whelan,[16] using 20 g l^{-1} starch in distilled water as substrate. Active extracts (10 µl) were mixed with 0.125 ml of 0.1 mol l^{-1} phosphate-citrate, 0.05 mol l^{-1} NaCl buffer, pH 5.5, and 0.125 ml of substrate was added. The mixture was incubated at 39 °C for 15 min. The reaction was stopped by the addition of 0.3 ml of 150 g l^{-1} $CuSO_4$ and the resulting solution was immediately heated at 100 °C for 20 min in order to allow the interaction between reducing sugars released from starch and copper ions (reduction of Cu^{2+} to Cu^{+}). The amount of reducing sugars released from starch was estimated by measuring the absorbance at 600 nm and comparing with a maltose standard. A blank without substrate and a control containing no enzymatic extract were run simultaneously. One unit of amylase activity was defined as the amount of enzyme releasing 1 µg maltose min^{-1} under the specified assay conditions.

Since diet particles (dehydrated alfalfa and milled corn grain) and microbial cells were both sonicated in the preparation of enzymatic extracts, the possible contribution of feeds to total amylase activity in extracts was evaluated using solutions of the feed components. Under the assay conditions, amylase activity in such solutions was negligible up to concentrations of 150 mg ml^{-1}, the highest amount that could be processed and solubilised. Prior to the assays the influence of anaerobiosis on ruminal amylase activity was assessed, developing comparative tests in two sets of tubes; the first set was kept open during the assays, and the second set was previously purged with CO_2 and then hermetically capped. Amylase activity was measured in both sets at 10 min intervals for a total time of 1 h, and no significant differences ($P < 0.05$; data not shown) were observed between the two treatments. In consequence, the rest of the determinations were carried out in open tubes. All the assays were carried out at least in triplicate.

Effect of different factors on amylase activity in ruminal extracts

Optimum pH for amylase activity was determined by incubation of the reaction mixture at pH values ranging from 2.0 to 12.0 using Stauffer's buffer.[17] Stability of amylase activity under different pH values was assessed by previous incubation of extracts at different pH values for 30, 60, 90 and 120 min. After this time, extracts were assayed for residual amylase activity at the optimum pH previously determined. Optimum temperature for amylase activity was determined in a range from 20 to 90 °C. Stability of amylases within this range of temperatures was assessed by previous incubation of extracts for 30, 60, 90 and 120 min at different temperatures and after measuring residual amylase activity as detailed previously.

Amylase inhibition assays

Effects of different substances of potential inhibitory effect (formic acid, boric acid, benzoic acid, lactic acid, formaldehyde, acetic acid, ascorbic acid, tannic acid, $SrCl_2$, $CuCl_2$, $SnCl_2$, $HgCl_2$, urea, $ZnSO_4$, $MgCl_2$, $CaCl_2$, NaCl, EDTA, EGTA, 1,10-phenanthroline and commercial plant α-amylase inhibitors from *Triticum aestivum* and *Phaseolus vulgaris*) on ruminal amylase activity were tested by previous incubation of extracts for 60 min at 25 °C in 0.1 mol l^{-1} phosphate-citrate, 0.05 mol l^{-1} NaCl buffer, pH 5.5, in the presence of the different substances. These compounds were chosen according to the literature, where chelators, divalent ions and naturally occurring α-amylase inhibitors in cereal and leguminous seeds showed some degree of inhibition on amylases from several non-ruminal sources.[18–23] Three different concentrations of the potential inhibitors were tested (5, 1 and 0.5×10^{-3} mol l^{-1}), except for tannic acid and plant α-amylase inhibitors (50, 10 and 5 ng per activity unit). Residual amylase activity was determined as previously described. Inhibition was expressed as a percentage, considering 100% the activity obtained when extracts were incubated without inhibitors.

Reversibility of inhibitory effects

The reversibility of the inhibitory effect produced by some compounds ($HgCl_2$, $SnCl_2$, $ZnSO_4$, formic acid, EDTA, EGTA and 1,10-phenanthroline) on amylase activity was tested by preparing an enzyme/inhibitor mixture that was divided into two aliquots. In one of them, inhibition was measured as detailed above and was used as a control; the other was previously dialysed for 24 h at 4 °C against 2000 volumes of 0.1 mol l^{-1} Tris, 0.05 mol l^{-1} $CaCl_2$ buffer, pH 6.8, and then assayed in the same way. Activities obtained in both cases were expressed as a percentage of those measured in extracts incubated without inhibitors. The reduction of the inhibitory effect observed when the activity measured in dialysed samples was compared with that of non-dialysed samples was expressed as 'recovered activity'.

SDS-PAGE zymograms

A new method of substrate SDS-PAGE zymograms for the detection and assignation of relative molecular masses of ruminal amylase-active fractions has recently been described.[15] Prior to electrophoretic separations, the effect of SDS on amylase activity was assessed by incubation of the extracts in SDS solutions (concentrations from 1 to 10 g l^{-1} and incubation times from 30 to 120 min). Even at maximum concentration (10 times that existing in the electrophoresis chamber) the activity was not significantly reduced (data not shown). Briefly, electrophoresis of enzyme preparations was carried out using 12% polyacrylamide, 0.25% copolymerised starch gels. Enzyme extracts and sample buffer (0.125 mol l^{-1} Tris, 200 g l^{-1} glycerol, 0.4 g l^{-1} bromophenol blue, 20 g l^{-1} sodium dodecyl sulphate) were mixed (1:1) but not boiled. About 10 μl of the mixture was added to each well. Electrophoresis was carried out in a first period for 30 min at a constant voltage of 30 V per gel, followed by a second 75 min period at a constant voltage of 100 V per gel. After the electrophoretic separation, gels were incubated in 0.1 mol l^{-1} phosphate-citrate buffer, pH 6.0, for 2–4 h at 39 °C. Finally, gels were washed and fixed in 120 g l^{-1} trichloroacetic acid (TCA) for 10 min and stained with 1 g l^{-1} lugol. Active bands were revealed as light yellow zones over a dark brown background of non-hydrolysed stained starch. Amylase-active fractions present in each rumen fraction were visualised by addition of the same number of activity units in each well after concentration of some of the extracts. Relative molecular mass determinations were performed by densitometry using specific software for gel image analysis (1D Image Analysis Software, Kodak Scientific Image Systems, New Haven, CT, USA).

Samples for zymograms showing inhibition of amylase activity were prepared by mixing the extracts with the inhibitor solutions, followed by 1 h of incubation at 25 °C, according to the procedure described by García-Carreño and Haard.[24] After mixing with sample buffer (1:1), about 25 μl of the final solution was loaded on each well of SDS-PAGE plates. All the wells contained the same amylase activity units, and extracts incubated without inhibitor were used as controls.

Statistical analyses

The data were analysed by one-way ANOVA followed by a comparison of means (Tukey's test). The percentages of inhibition were normalised using $\sin^{-1}$ transformation of their square root.[25] All statistical analyses were conducted using Statgraphics Plus 4.0 software (Statistical Graphics Corp, Rockville, MD, USA).

RESULTS

Distribution of amylase activity

Total amylase activity measured in ruminal extracts, as well as the distribution of this activity in the different semi-purified fractions, is shown in Table 1. Most of the amylase activity was detected either in the particulate fraction (70%) or in the bacterial fraction (21%), being very low in the extracellular fraction (less than 10%). It was decided that extracts obtained from the particulate fraction could be representative, both quantitatively and qualitatively, of the amylase activity present in the rumen. This fraction was therefore selected for the rest of the assays.

Optimum conditions for the assays

Results obtained after determination of amylase activity present in the aforementioned fraction under different pH and temperature conditions showed that the optimum pH was 5.5. Sensitivity of amylase activity to previous incubation at different pH values indicated that most of the activity was retained when extracts were pre-incubated in the pH range from 5.0 to 8.0, but a marked reduction (80% or more) was measured at extreme pH values (2.0 and 12.0). In relation to temperature, the highest amylase activity was observed in the range between 45 and 60 °C, with a maximum at 50 °C. Almost 60% of total activity was retained after 1 h of pre-incubation at 60 °C, but temperatures of 70 °C or higher, even after a short time of incubation, markedly reduced amylase activity in extracts.

Inhibition of amylase activity

The effect of different inhibitors on amylase activity determined in the particulate fraction of ruminal extracts is shown in Table 2. The maximum concentration assayed (5×10^{-3} mol l^{-1}) produced a high inhibitory effect (from 60 to 90% of total activity) by salts of some divalent ions (Sn^{2+}, Hg^{2+} and Cu^{2+}). Only one organic compound (tannic acid) produced a comparable inhibition (79%). A lower inhibitory effect (from 15 to 50% of total activity) was produced mainly by organic compounds such as formic, benzoic and ascorbic acid, as well as by chelating agents such as EDTA, 1,10-phenanthroline and EGTA. Negligible inhibition (less than 10%) was detected when the rest of the compounds were assayed. When the potential inhibitors were examined at a much lower relative concentration (5×10^{-4} mol l^{-1}), the general trends previously described were maintained, but inhibition values were significantly decreased in most cases (Table 2). This reduction was less noticeable in the assay performed using Zn^{2+}, which produced 26% inhibition even at such a low concentration.

The persistence of the inhibitory effect in some of the assayed compounds, tested after extensive dialysis of the mixtures containing both the enzyme and inhibitors, is shown in Table 3. A marked reduction was observed in the inhibitory effect produced by Hg^{2+} and Sn^{2+} (and then an increase in the activity recovered). In contrast, a persistence of the inhibitory effect, as shown by the very low values of recovered activity after dialysis, was measured for Zn^{2+} and the chelating agents.

Table 1. Distribution of amylase activity in rumen contents. Results are mean of three determination ± SD

Fraction	*% of total activity*
Particle-associated fraction	70.0 ± 6.75a
Bacterial fraction	21.4 ± 6.25b
Extracellular fraction	8.6 ± 6.07c

Values sharing a letter are not significantly different ($P < 0.05$).

Electrophoretic assays

SDS-PAGE zymograms of amylase activity present in the three assayed ruminal fractions showed important differences in the pattern of bands (Fig 1, lanes 1–3). Several active fractions were found in extracts obtained from the particulate fraction, being lower in the bacterial fraction and almost absent in the extracellular fraction. In the first case, molecular masses ranged from 36 to more than 100 kDa. A higher intensity was observed in bands corresponding to relative molecular masses of 36, 54, 70, 85 and 94 kDa, as well as in those of more than 100 kDa.

When the same amount of amylase activity units was added to the lanes (Fig 1, lanes 4–6), the three assayed fractions showed differences in their electropohoretic pattern. The particle-associated fraction (fraction 1) included those bands found in the two other studied fractions, although the former fraction showed a higher number of different amylolytic bands. The zymogram obtained after incubation of ruminal extracts of the particulate fraction in the presence of different inhibitors is shown in Fig 1, lanes 7–10. No effect of urea (lane 7) or commercial wheat α-amylase inhibitor (lane 8) was detected, but incubation with $ZnSO_4$ resulted in marked inhibition (lane 9). $SnCl_2$ (lane 10) caused total inhibition of amylase activity.

DISCUSSION

The possibility of reducing starch degradation in the rumen by selective inhibition of amylases remains virtually unexplored and could be an alternative way to reduce negative effects resulting from digestion of starch-rich diets, as well as to increase intestinal utilisation of this nutrient. Such an inhibitory effect should present several features: (a) inactivation of a substantial part of the amylase activity present in the rumen; (b) inhibition produced by a non-toxic substance; (c) the effect must be achieved at very low relative concentration of the potential inhibitor in order to minimise possible secondary effects; and (d) it should be stable and persistent under changing conditions in the rumen.

In this paper, different compounds were evaluated

Inhibitor	Concentration (mol l^{-1})		
	5×10^{-3}	1×10^{-3}	5×10^{-4}
Formic acid	47.8±3.85d	14.3±3.56d	6.6±1.08
Boric acid	6.0±2.93	9.4±2.63	3.2±1.82
Benzoic acid	36.8±1.54de	26.4±3.79c	4.7±2.33
Lactic acid	8.2±2.23	6.1±3.22	7.1±0.91
Formaldehyde	14.3±0.87f	7.8±1.23	7.8±5.35
Acetic acid	9.4±8.60	13.8±4.16d	16.7±8.99cd
Ascorbic acid	43.6±5.18de	17.1±2.36d	10.9±1.13de
Tannic acid[a]	79.2±3.86b	13.1±3.47d	12.8±3.42de
$SrCl_2$	8.5±5.99	11.6±9.75d	12.9±5.75de
$CuCl_2$	65.3±3.39c	10.5±7.90d	8.0±3.10
$SnCl_2$	90.2±4.89a	28.8±6.36bc	30.4±7.50b
$HgCl_2$	82.1±5.92ab	43.5±3.21a	49.9±6.74a
Urea	5.3±8.24	5.6±2.77	4.1±2.01
$ZnSO_4$	44.4±4.85de	34.8±3.07b	32.7±9.97bc
$MgCl_2$	−3.1±7.32	0.7±4.10	−2.6±4.21
$CaCl_2$	−2.8±7.20	0.4±6.32	0.0±4.45
NaCl	−2.7±5.83	2.8±4.41	−2.7±8.21
EDTA	36.9±6.04de	5.3±8.07	6.4±8.67
EGTA	30.3±5.28e	12.4±5.49d	10.9±7.65de
1, 10-Phenanthroline	32.5±7.21e	9.4±3.56	6.6±3.07
Triticum inhibitor[a]	3.0±0.45	3.2±3.56	4.3±1.07
Phaseolus inhibitor[a]	4.4±2.14	5.2±2.31	4.1±1.97

Table 2. Effect of different substances on amylase activity of particle-associated ruminal extracts. Results are expressed as percentage of inhibition relative to extracts with no inhibitors. Data are mean of three determinations ± SD

[a] Final concentrations of 50, 10 and 5 ng per activity unit.
Values sharing letters within each column are not significantly different ($P<0.05$).Substances that produced inhibition below 10% were not included in the statistical analysis for clarity of presentation.

from this perspective. Some of them, eg urea, acetic acid and plant α-amylase inhibitors, did not produce significant inhibition of amylase activity under the assayed conditions. Inhibition produced by organic acids was more noticeable, but they could modify the ruminal ecosystem through excessive acidification, mainly when the rumen is in pre-acidosis status. However, some organic acids have been described as acidosis-reducing agents owing to their interference in metabolism and utilisation of lactate by ruminal microbiota.[26] On the other hand, commercial plant α-amylase inhibitors seem to be more effective on enzymes produced by eukariotes, although some bacterial amylases have also shown susceptibility to plant α-amylase inhibitors.[19,20] In contrast, rumen amylases were found to be particularly sensitive to divalent ions such as Hg^{2+}, Sn^{2+}, Cu^{2+} and Zn^{2+}. The inhibitory effect of such ions on amylases of non-ruminal origin, such as plant[23] and microbial[18,21] amylases, has been described previously, but toxicity was produced even by low concentrations of them, with the exception of Zn^{2+} ions.[27] Moreover, zinc, mainly in the form of Zn-methionine supplements, has been reported to improve animal performance.[28–30] Considering the results obtained in the present work, it is suggested that one of the factors involved in such a

Table 3. Effect of $CaCl_2$ dialysis on recovery of amylase activity. Extracts were previously incubated in the presence of three concentrations of the different assayed substances with potential inhibitory effect. Data are mean of three determinations ± SD

Inhibitor	% amylase activity[a]					
	5×10^{-3} mol l^{-1}		1×10^{-3} mol l^{-1}		5×10^{-4} mol l^{-1}	
	Before dialysis	After dialysis	Before dialysis	After dialysis	Before dialysis	After dialysis
Formic acid	52.2±3.85a	70.2±2.39b	85.7±3.56a	90.0±1.14a	93.4±1.08a	94.3±1.30a
$SnCl_2$	9.8±3.89a	72.3±1.21b	56.5±3.21a	82.7±0.66b	69.6±7.50a	89.1±1.10b
$HgCl_2$	17.9±5.92a	82.8±1.82b	43.4±2.77a	89.7±2.08b	50.1±6.74a	93.3±1.55b
$ZnSO_4$	55.6±4.85a	64.4±0.60b	74.3±4.10a	73.2±1.92a	74.0±2.53a	80.5±2.41b
EDTA	63.1±6.04a	90.2±3.75b	87.6±5.49a	91.3±3.02a	93.6±8.67a	97.0±2.20a
EGTA	69.7±5.28a	80.6±1.75b	90.6±3.56a	93.1±1.89a	89.1±7.65a	94.4±4.11a
1,10-Phenanthroline	67.6±7.21a	86.3±1.69b	90.3±3.45a	95.1±3.94a	93.4±3.07a	98.7±3.81a

[a] Data are expressed as percentage of amylase activity, considering 100% the activity of the control assayed without any inhibitor.
Values sharing a letter within each inhibitor and concentration are not significantly different ($P<0.05$).

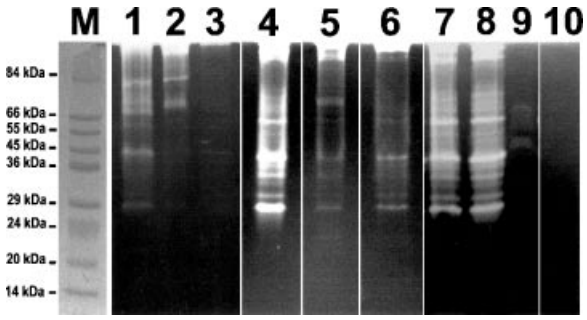

Figure 1. Substrate SDS-PAGE of amylase activity in ruminal extracts: lane M, molecular mass markers; lane 1, particle-associated amylases; lane 2, bacterial fraction-associated; lane 3, extracellular fraction; lanes 4–6, same as lanes 2–4 respectively but using equal amounts of amylase activity units; lanes 7–10, extracts were mixed with the inhibitors indicated at the concentrations detailed in the text—lane 7, urea; lane 8, wheat α-amylase inhibitor; lane 9, $ZnSO_4$; lane 10, $SnCl_2$.

positive effect could be its influence on the digestion of starch, by increasing intestinal availability of the substrate. The relatively high inhibitory effect produced by this ion on rumen amylases (44%) is in agreement with other findings pointing to Zn^{2+} as a strong inhibitor for α-amylases,[21,23] and this seems to be a general feature of amylases from anaerobic bacteria.[18]

All the assayed substances decreased their inhibitory effect when their relative concentrations were reduced from 5×10^{-3} to 5×10^{-4} moll^{-1} (Table 2). However, $ZnSO_4$ maintained a relatively high inhibition (25%) at the lowest concentration assayed (5×10^{-4} moll^{-1}). This fact, together with its low toxicity for ruminants,[27] could make Zn^{2+} a suitable modulator of enzymatic degradation of starch in the rumen. The specificity of the inhibition, as well as its persistence under the changing conditions existing in the rumen, was tested by dialysing against Ca^{2+} extracts on which amylase activity had previously been inhibited by some substances. Although the role of Ca^{2+} still remains unclear, it is widely recognised that it is necessary for the activity of amylases.[31–33] Results presented in Table 3 showed that, in all cases, Ca^{2+} was able to displace the other substances and, as a result, most of the amylase activity was recovered after dialysis. This suggests a lack of specificity in the inhibition produced by the assayed substances. Nevertheless, this effect was less noticeable in the case of Zn^{2+}, thus pointing to the existence of a more stable link between this cation and the active enzymes. In addition, dialysis of samples (1 ml each) against a volume 2000 times greater simulated to a certain extent the dilution taking place in the rumen as a result of the continuous changes in volume produced by feed intake and digesta outflow. The persistence of a certain inhibition under such circumstances would be a desirable feature in order to guarantee a reduction in starch degradation by ruminal amylases. Taking into account all the previous results, $ZnSO_4$ was the substance that best fulfilled the previously described requisites for an effective modulation of enzymatic digestion of starch.

Electrophoretic studies of ruminal enzymatic activities are scarce, and there are few references in the literature to zymograms of rumen amylases,[34] possibly because of the difficulties in revealing the low activity of these enzymes. Nevertheless, in the present work the use of an improved method[15] clearly revealed amylase-active bands as yellow zones over a stained background of non-hydrolysed starch. The resolution obtained also allowed quantification of the relative molecular masses of the different bands by comparison with a standard molecular weight marker, as well as visualisation of the inhibition (Fig 1, lanes 7–10). Although the average mass of most studied amylases is around 50 kDa, zymograms showed the presence of a certain number of active bands with different molecular masses ranging from 36 to more than 100 kDa. Previous reports have shown apparent molecular masses of amylases from ruminal origin of 92 kDa[8] and 77 kDa.[35] However, greatly different values have also been reported for amylases from different bacterial origins.[33,36] The pattern of bands obtained should reflect the diversity of micro-organisms in the rumen that exhibit amylase activity, although the presence of multiple bands may also be related to the presence of isozymes, a common feature described for anaerobic bacteria degrading polysaccharides.[18] Since not all bacteria are equipped with a complete array of enzymes,[37] the digestion of starch in the rumen should require integration among bacterial species in a cooperative effect of enzymatic mechanisms.[38] Inhibition affected most of the isoforms present in the extracts, suggesting the existence of a common mechanism of action for most of them.

Although the inhibition of amylases represents an alternative approach to those procedures aimed at reducing ruminal starch digestion by a previous physical or chemical modification of the substrate,[3,11,39,40] results obtained in the present work show that, owing to the diversity of amylases present in the ruminal ecosystem, a complete and specific inhibition seems to be quite unlikely. Nevertheless, in recent years there has been considerable interest in the use of trace minerals in ruminant diets, this being stimulated by reports indicating improved performances,[28,29] but the mode of action of trace minerals such as Zn^{2+} is virtually unknown.[41] The results obtained in the present work indicate that Zn^{2+} is a promising agent in the regulation of the enzymatic degradation of starch taking place in the ruminal ecosystem. However, the possibility of differences in the inhibitory effect of this ion on the amylase activity of lysed and unlysed microbial cells must be evaluated. Thus further research is necessary mainly to check the *in vivo* effects of Zn^{2+} in preventing acidosis, as well as to measure its effects on the efficiency of utilisation of readily fermentable starches.

ACKNOWLEDGEMENTS

The authors acknowledge the facilities and the animals

provided by 'Patronato Rodríguez Penalva' (Huéscar, Granada). TFM acknowledges the financial support by the Consejería de Educación (Junta de Andalucía).

REFERENCES

1 Waldo DR, Extent and partition of cereal grain starch digestion in ruminants. *J Anim Sci* **37**:1062–1074 (1973).
2 Huntington GB, Starch utilisation by ruminants: from basics to the bunk. *J Anim Sci* **75**:852–867 (1997).
3 Theurer CB, Grain processing effects on starch utilisation by ruminants. *J Anim Sci* **63**:1649–1662 (1986).
4 Williams AG, Withers E and Coleman GS, Glycoside hydrolases of rumen bacteria and protozoa. *Curr Microbiol* **10**:287–294 (1984).
5 Chesson A and Forsberg CW, Polysaccharide degradation by rumen microorganisms, in *The Rumen Microbial Ecosystem*. Ed by Hobson PN, Elsevier Science, London, pp 251–284 (1988).
6 Jouany JP, Demeyer DI and Grain J, Effect of defaunating the rumen. *Anim Feed Sci Technol* **21**:229–265 (1988).
7 Mendoza GD, Britton RA and Stock RA, Influence of ruminal protozoa on site and extent of starch digestion and ruminal fermentation. *J Anim Sci* **71**:1572–1578 (1993).
8 McWethy SJ and Hartman PA, Purification and some properties of an extracellular alpha-amylase from *Bacteroides amylophilus*. *J Bacteriol* **129**:1537–1544 (1977).
9 Owens FN, Zinn RA and Kim YK, Limits to starch digestion in the ruminants small intestine. *J Anim Sci* **63**:1634–1648 (1986).
10 Sauvant D, Chapoutot P and Archimede H, La digestion des amidons par les ruminants et ses conséquences. *INRA Prod Anim* 7:115–124 (1994).
11 McAllister TA, Cheng KJ, Rode LM and Buchanan-Smith JG, Use of formaldehyde to regulate digestion of barley starch. *Can J Anim Sci* **70**:581–589 (1990).
12 Bae HD, McAllister TA, Yanke J, Cheng KJ and Muir AD, Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. *Appl Environ Microbiol* **59**:2132–2138 (1993).
13 Slyter LL, Influence of acidosis on rumen function. *J Anim Sci* **43**:910–929 (1976).
14 Sauvant D, Meschy F and Mertens D, Les composantes de l'acidose ruminale et les effets acidogènes des rations. *INRA Prod Anim* **12**:49–60 (1999).
15 Martínez TF, Alarcón FJ, Díaz M and Moyano FJ, Improved detection of amylase activity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with copolymerized starch. *Electrophoresis* **21**:2940–2943 (2000).
16 Robyt JF and Whelan WJ, Reducing value methods for maltodextrins: I. Chain-length dependence of alkaline 3,5-dinitrosalicylate and chain-length independence of alkaline copper. *Anal Biochem* **45**:510–516 (1972).
17 Stauffer C, *Enzyme Assays for Food Scientists*. Van Nostrand Reinhold/AVI, New York, Chap 19 (1989).
18 Antranikian G, Physiology and enzymology of thermophilic anaerobic bacteria degrading starch. *FEMS Microbiol Rev* **75**:201–218 (1990).
19 Orlando AR, Ade P, Di Maggio D, Fanelli C and Vittozzi L, The purification of a novel amylase from *Bacillus subtilis* and its inhibition by wheat proteins. *Biochem J* **209**:561–564 (1983).
20 Silano V, Alpha-amylase inhibitors, in *Enzymes and Their Role in Cereal Technology*, Ed by Kruger IE, Lineback D and Stauffer CE, American Association of Cereal Chemists, St Paul, MN, pp 141–199 (1987).
21 Chiang JP, Altar JE and Sterberg M, Purification and characterization of a thermostable alpha-amylase from *Bacillus licheniformis*. *Starch* **31**:86–92 (1979).
22 Irshad M, Goel M and Sharma CB, Effect of Zn^{2+} on plant α-amylases *in vitro*. *Phytochemistry* **20**:2123–2126 (1981).
23 Berbezy P, Legendre L and Maujean A, Purification and characterization of alpha-amylase from vine shoot inter-nodes. *Plant Physiol Biochem* **34**:353–361 (1996).
24 García-Carreño FL and Haard NF, Characterisation of protease classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. *J Food Biochem* **17**:97–113 (1993).
25 Sokal R and Rohlf JF, *Biometry: the Principles and Practice of Statistics in Biological Research*, 2nd edn. Freeman, San Francisco, CA (1981).
26 Carro MD, López S, Valdés C and Ovejero FJ, Effect of DL-malate on mixed ruminal microorganism fermentation using the rumen simulation technique (RUSITEC). *Anim Feed Sci Technol* **79**:279–288 (1999).
27 Underwood EJ, *Trace Elements in Human and Animal Nutrition*. Academic Press, New York (1977).
28 Spears JW, Zinc methionine for ruminants: relative bioavailability of zinc in lambs and effects on growth and performance of growing heifers. *J Anim Sci* **67**:835–843 (1989).
29 Kellog DW, Rakes JM and Gliedt DW, Effect of zinc methionine supplementation on performance and selected blood parameters of lactating dairy cows. *Nutr Rep Int* **40**:1049–1055 (1989).
30 Puchala R, Sahlu T and Davis JJ, Effects of zinc-methionine on performance of Angora goats. *Small Ruminant Res* **33**:1–8 (1999).
31 Antranikian G, Microbial degradation of starch, in *Microbial Degradation of Natural Products*, Ed by Winkelmann G, VCH Publishers, New York, pp 27–56 (1992).
32 Whitaker JR, *Principles of Enzymology for the Food Sciences*. Marcel Dekker, New York, pp 391–423 (1994).
33 Robyt JF and Whelan WJ, The alpha-amylases, in *Starch and Its Derivatives*, Ed by Radley A, Chapman and Hall, London, Chap 14, pp 430–476 (1968).
34 Whitehead R and Cotta MA, Identification of intracellular amylase activity in *Streptococcus bovis* and *Streptococcus salivarius*. *Curr Microbiol* **30**:143–148 (1995).
35 Freer SN, Purification and characterisation of the extracellular alpha-amylase from *Streptococcus bovis* JB1. *Appl Environ Microbiol* **59**:1398–1402 (1993).
36 McTigue MA, Kelly CT, Doyle EM and Fogarty WM, The alkaline amylase of the alkalophilic *Bacillus* sp. IMD 370. *Enzyme Microb Technol* **17**:570–573 (1995).
37 Kotarski SF, Waniska RD and Thurn KK, Starch hydrolysis by the ruminal microflora. *J Nutr* **122**:178–190 (1992).
38 Cotta MA, Interaction of ruminal bacteria in the production and utilisation of maltooligosaccharides from starch. *Appl Environ Microbiol* **58**:48–54 (1992).
39 Ørskov ER, The effect of processing on digestion and utilisation of cereals by ruminants. *Proc Nutr Soc* **35**:245–256 (1976).
40 Campling RC, Processing cereal grains for cattle: a review. *Livest Prod Sci* **28**:223–234 (1991).
41 Spears JW, Organic trace minerals in ruminant nutrition. *Anim Feed Sci. Technol* **58**:151–163 (1996).