Activity and stability of a fungal 1,4-beta-endo-xylanase preparation in the rumen of sheep

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ABSTRACT

Experiments were conducted with 1-3 years old, rumen cannulated Merino wethers to characterize the stability of the 1,4-β-endo-xylanase activity of a commercially available enzyme preparation (Rumino-Zyme, 250 FXU/g xylanase activity, Dr. Bata Ltd., Hungary). The Remazol blue technique used for determination of the xylanase activity of the rumen fluid and solid samples proved simple, quick and reliable.

The xylanase activity in the rumen of fasting wethers was found to be 20-30 FXU/l. It was by about 2.5 times greater two and four hours after feeding than the value measured pre-feeding. Exogenous xylanase added directly into the rumen of fasted wethers caused an increase in the enzyme activity. After the peak activity at 5 min after treatment the enzymatic activity decreased with an estimated half-life of 45-50 min. This result of the present investigation did not support the earlier observations, which indicated lasting activity of external NSP-enzymes *in vivo*.

KEY WORDS: xylanase, *Thermomyces lanuginosus*, Remazol blue

INTRODUCTION

Non-starch polysaccharidase enzymes (NSP-enzymes) are known to improve the utilization of nutrients of feed rations first of all in monogastrics. Ruminants

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digest non-starch polysaccharides due to the variety of enzymes produced by the rumen microflora (Annison and Bryden, 1998). External fibre degrading enzymes are believed to increase the availability of nutrient substances for further processing into microbial proteins and volatile fatty acids (VFAs) in the rumen fluid. Silage additives are important application of external fibrolytic enzymes in the ruminant nutrition. Authors reported improved digestibility of forages, and enhanced weight gain in beef cattle (Nakashima and Ørskov, 1989; Beauchemin et al., 1995) and improved dry matter intake, increased milk and milk protein production in dairy cattle (Chamberlain and Robertson, 1992; Stokes, 1992; Kung et al., 2000). Enzymatic treatment decreased the pH, xylose and sugar content of the silage (Stokes, 1992).

By using a Rumen Simulation Technique (RuSiTec) Wang et al. (2001) reported that exogenous NSP-enzymes (cellulase and xylanase) improved the utilization of the neutral detergent fibre, increased the quantity of reducing sugars and VFA, elevated the count of cellulolytic bacteria and enhanced the rate of production of microbial proteins. On this basis, one may expect that due to the increased quantity of available proteins and energy (in form of VFA) additional (dietary) NSP-enzymes might improve the production efficiency of dairy or beef cattle as in fact was reported (Nussio et al., 1997; Lewis et al., 1999; Schingoethe et al., 1999; Brydl et al., 2001; Jurkovich et al., 2002). Early studies suggested that supplemental NSP-enzymes were rapidly degraded in the rumen due to the proteinase activity of microflora. On the contrary Hall et al. (1993) published that the endoglucanase from *C. thermocellum* was completely resistant to inactivation by small intestine proteinases in mice. Fontes et al. (1995) incubated NSP-enzymes for 180 min at 37°C in presence of bovine α-chymotrypsin and porcine pancreatin. Cellulase and xylanase enzymes proved to preserve their activity along the treatment, while the half-life of endoglucanase decreased to 10 and 70 min, respectively. In their *in vitro* experiments Hristov et al. (1998) demonstrated also remarkable resistance of carboxymethyl-cellulase (CMC-ase), xylanase, β-glucanase to microbial fermentation, viz. no significant decline in enzyme activity was observed in the first 6 h of incubation. Their *in vivo* experiments showed that peaks in CMC-ase, xylanase, β-glucanase activities were reached within 1.5 h after treatment with a remarkable stability thereafter. Morgavi et al. (2000) incubated four commercially available preparations of fibrolytic enzymes *in vitro* with rumen digesta of sheep. NSP-enzymes showed different stability. CMC-ase and xylanase from the fungus *A. niger* was stabile for over 6 h, while β-glucosidase and β-xylosidase were much more labile. Rate of degradation of enzymes depended also on the kind of the preparation.

The objective of our investigation was to measure the xylanase activity in the rumen and to characterize the stability of the 1,4-β-endo-xylanase activity of a commercially available enzyme preparation (Rumino-Zyme, 250 FXU/g xylanase activity, Dr. Bata Ltd., Hungary).

MATERIAL AND METHODS

Trial 1

The goal of the experiment was to gain data on the xylanase activity of the rumen fluid of sheep kept on a ration of high structural fibre. In this experiment 8 one-year-old, rumen cannulated (Hecker, 1974) Merino wethers of 40 kg average liveweight were used. The sheep were kept individually in metabolic cages within reach of sight and auditory communication. The animals were fed two times a day (at 8.00 a.m. and at 4.00 p.m.) with a ration of 400 g meadow hay and 250 g lamb concentrate (4.65 MJ MEm and 153 g crude protein daily). Licking salt and water were offered *ad libitum*. In this part of the experiment no enzyme preparation was added to the ration. After two weeks of preliminary period, two times a week (on Wednesdays and Fridays) 50 ml of rumen fluid samples were taken from each sheep just before the morning feeding and 2 and 4 h thereafter. The samples were kept in thawing ice until measuring the xylanase activity. In one occasion a bigger quantity (200 ml) of solid material was taken from the rumen right before the morning feeding in order to measure enzyme activity bound to the solid fibre phase.

Trial 2

The goal of the trial was to collect base line data on the enzyme activity of the rumen fluid in fasting sheep. Four, 3 years old, rumen cannulated Merino wethers of about 70 kg average liveweight were used to obtain rumen fluid samples 16, 17.5 and 19 h after the last feeding. In this trial the sheep were fed only once a day (at 4.00 p.m.) with a ration of 700 g lucerne hay and 400 g sheep concentrate (7.49 MJ NEm, 125 g crude protein).

Trial 3

With this experiment we attempted to follow up the activity change of the exogenous 1,4-ß-endo-xylanase enzyme added directly into the rumen at a quantity sufficient to rise the total extracellular xylanase activity of the rumen fluid. In this experiment the sheep of trial 2 were used with the same feeding regime and accommodation. At 8.00 a.m. (viz. 16 h after the last feeding) a base-line rumen fluid sample was taken from each sheep. Afterwards a single dose of 10 g enzyme preparation of 250 FXU/g 1,4-ß-endo-xylanase activity dissolved in 100 ml of water was poured into the rumen *via* the cannula. Further samples were taken 5, 10, 15, 30, 45, 60, 90, 120 and 180 min after application of the enzyme preparation.

Experimental material (enzyme preparation)

A thermophilic fungus, *Thermomyces lanuginosus*, known to produce cellulase free extracts high in xylanase and low in β-xylosidase, β-glucosidase and α-arabinosidase activity (Purkarthofer and Steiner, 1995; Bennett et al., 1998) was used to produce an enzyme preparation as reported elsewhere (Kutasi et al., 2001). The obtained product (Rumino-Zyme) is light-brown, granulated material (particle size: 400-500 μm) of 90% dry matter content, which contains thermally resistant endoxylanase from the fungus *Thermomyces lanuginosus*. The 1,4-β-endo-xylanase preserves its activity within the range of pH 4.5-8.0 and 30-50°C. Shelf life at 20°C is longer than 6 months. Enzyme activity of the product is 250 FXU/g (FXU: one unit of xylanase activity was expressed as μmol of Remazol xylan-degradation products released in one min).

Determination of the enzyme activity in the rumen fluid and solid parts

Xylanase activity was measured in the supernatant of rumen fluid samples and also in the solid materials of the rumen samples. Ten ml of rumen fluid was centrifuged for 15 min at 5000 rpm and the supernatant was used for further measurements. In the preparation of the solid fraction sample of rumen fluid (100 ml) was sieved through double layers of gaze. Particles were collected from the gaze and its suspension with 500 ml of 0.05 mol phosphate buffer (pH 6.5) was stirred with a magnetic stirrer for 30 min. The suspension was filtrated again through layers of gaze. The solid part was collected and suspended again in 50 ml phosphate buffer. The suspension was mechanically shaken in a homocentric equipment at 30°C for 30 min in order to activate the enzyme. The activated suspension was sieved through a filter paper (MN 615 ¼) until a water-clear fluid was obtained. This filtered material was used for further measurements. The weight of the wet material withheld by the filter paper was taken and then dried until steady weight. Enzyme activity was calculated for the dry weight.

Measurement of the enzyme activity

Ten mg substrate (4-O-methyl-D-glucorono-D-xylan stained with Remazol blue, Fluka 66960) was put into test-tube and 2.0 ml of test material (rumen fluid supernatant, or filtrate of the rumen solid material) were added. After 120 min incubation at 50°C, 5 ml of stop reagent (150 ml ethanol + 1 ml 1 N HCl) was added to the medium and stirred for 1 min and left standing at room temperature for 15 min and then centrifuged for 10 min at 3 000 rpm. The absorbance was measured at 590 nm wavelengths against blank that contained phosphate buffer. Colour intensity of the liberated Remasol blue was measured and expressed in FXU units.

The standard series consisted of from 0.01 to 0.15 mg *Trichoderma viridis* (Fluka 95595) xylanase of 2.5 FXU/mg activity.

Statistical analysis

Data were analysed by variance analysis and linear regression with the SPSS 8.0 for Windows software.

RESULTS AND DISCUSSION

Our studies showed that xylanase activity can be measured with reliable efficiency in the Remazol stained xylane system.

In Trial 1 the xylanase activity before the morning feeding was low (Figure 1) and increased considerably (from 46.3 to 114.8 and 111.7 FXU/l; $P<0.001$) following the feed intake. This finding of our experiment is in accordance with the data reported by Huhtanen and Khalili (1992) and Michalet-Doreau et al. (2001).
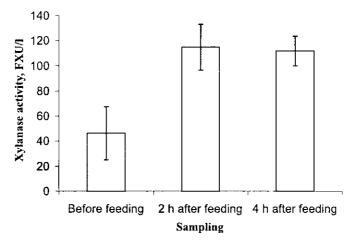

Figure 1. Xylanase activity in the rumen fluid before and after feeding

For the time being it is impossible to measure separately the activity of the internal and exogenous (experimentally added) NSP-enzymes. It follows that enzymes produced by the rumen microorganisms may shadow the activity of the external enzymes and may lead to considerable misinterpretation of the data. Therefore for studying the activity of external NSP-enzymes in *in vivo* systems the careful design of time regime in relation with feeding is of vital importance.

Fibre-bound enzyme activity was higher than that in the liquid fraction. However, in our experiment enzyme activity of the solid rumen particles is of limited value in characterizing the xylanase activity of the rumen system due the high standard deviations of the data. Sonication might be better than mechanical stirring to release xylanase activity from the crude plant particles. But in our study analysis of rumen fluid samples can set firm basis for the estimation of the xylanase activity in the rumen.

In Trial 2 the xylanase activity of the rumen fluid was low after about 16 h of the last feeding (in a state of "fasting") with low standard deviation and decreased slowly in the function of time (Figure 2). These data indicate that for characterizing the base-line xylanase activity of the rumen the samples should be taken several hours after feeding, because in these times the xylanase activity of the rumen micro-organisms is very low and steady.
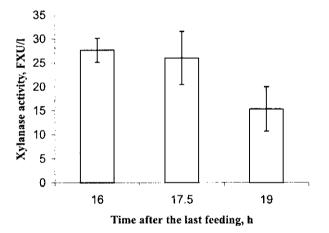
Figure 2. Xylanase activity in the rumen fluid in fasting sheep

In Trial 3 the exogenous xylanase enzyme activity in the rumen fluid was about 250 FXU/l. This additional enzyme increased the base-line xylanase activity of the rumen fluid by about 300 % within 5 min after treatment (Figure 3) from 50 FXU/l to 161 FXU/l (P<0.001). The average recovery rate of the original enzyme activity was about 54%. A significant part of the xylanase activity in the rumen is associated with crude plant particles. This may explain incomplete recovery of added xylanase. The additional enzyme preserved 63% of its activity for 45 min (120 FXU/l). After 60 and 90 min the original enzyme activity decreased down to 41 (96 FXU/l) and 34% (88 FXU/l), respectively. Between 90 and 120 min after treatment the enzyme activity became stabilized over 30% of the original activity.
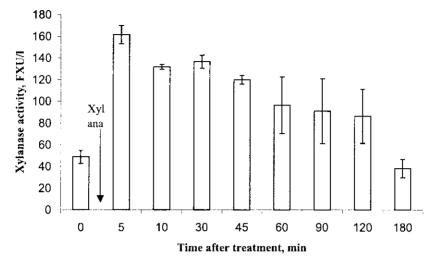
Figure 3. Xylanase activity in the rumen fluid prior to and after xylanase treatment

Three hours after treatment no increment in the enzymatic activity was seen and the activity returned back to the initial values (38 FXU/l).

The data (Figure 3) indicate significant rise in the xylanase activity which support the findings of several authors (Hall et al., 1993; Fontes et al., 1995; Hristov et al., 1998; Morgavi et al., 2000, 2001; Wang et al., 2001). In our experiments, however, the increased activity was observed for about two hours only. Hristov et al. (1998) in their 15 h *in vivo* experiments found peak enzymatic activity in the rumen 1.5 h after intra-rumen application of the enzyme preparation. No data were published about the time when the activity of the external enzyme ebbed away and in their paper no information was found about the origin (microbial or fungus) of the enzyme.

We concluded, that dietary (exogenous) 1,4-β-endo-xylanase enzyme disintegrates in the rumen within short after treatment with an estimated half-life of about 45-50 min. It follows that substantial improvement in rumen digestibility of non-starch polysaccharides can be expected only from those additives, which provides long lasting enzymatic activity in the rumen.

REFERENCES

Annison E.F., Bryden W.L., 1998. Perspectives on ruminant nutrition and metabolism. I. Metabolism in the rumen. Nutr. Res. Rev. 11, 173-198

Beauchemin K.A., Rode L.M., Sewalt V.J., 1995. Fibrolytic emzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75, 641-644

Bennet N.I., Ryen J., Biely P., Vrasanska M., 1998. Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus ATCC 46882*. Carbohydr. Res. 306, 445-455

Brydl E., Rafai P., Könyves L., Jurkovich V., Tegzes L., Nagy G., Kutasi J., Bata Á., 2001. NSP enzymes help rumen microbes. Feed Mix 9, 22-26

Chamberlain D.G., Robertson S., 1992. The effects of the addition of various enzyme mixtures on the fermentation of perennial ryegrass silage and on its nutritional value for milk production in dairy cows. Anim. Feed Sci. Tech. 37, 257-264

Fontes C.M.G.A., Hall J., Hirst B.H., Hazlewood G.P., Gilbert H.J., 1995. The resistance of cellulases and xylanases to proteolytic inactivation. Appl. Microbiol. Biotechnol. 43, 52-57

Hall J., Ali S., Surani M.A., Hazlewood G.P., Clark A.J., Simons J.P., Hirst B.H., Gilbert H.J., 1993. Manipulation of the repertoire of digestive enzymes into the gastrointestinal tract of transgenic mice. Bio/Technol. 11, 376-379

Hecker J.F., 1974. Experimental Surgery on Small Ruminants. Butterworth Co. Ltd., London, pp. 96-150

Hristov A.N., McAllister T.A., Cheng K.J., 1998. Stability of exogenous polisaccharide-degrading enzymes in the rumen. Anim. Feed Sci. Tech. 76, 161-168

Huhtanen P., Khalil H., 1992. The effect of sucrose supplements on particle associated carboxymethylcellulase (EC 3.2.1.4) and xylanase (EC 3.2.1.8) activities in cattle given grass-silage-based diet. Brit. J. Nutr. 67, 245-255

Jurkovich V., Brydl E., Rafai P., Könyves L., Tegzes L., Kutasi J., Bata Á., Nagy G., Bartyik J., Fülöp A., 2002. The effects of a non-starch polysaccharidase enzyme preparation from *Thermomyces lanuginosus* on the ruminal volatile fatty acid production, energy and protein metabolism and milk yield of dairy cattle. Acta Vet. Acad. Sci. Hung. (in press)

Kung L., Treacher R.J., Nauman G.A., Smagala A.M., Endres K.M., Cohen M.A., 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. J. Dairy Sci. 83, 115-122

Kutasi J., Bata Á., Brydl E., Rafai P., Jurkovich V., 2001. Characterisation and effects of a xylanase enzyme preparation extracted from *Thermomyces lanuginosus* cultures. Acta Vet. Acad. Sci. Hung. 49, 175-184

Lewis G.E., Sanchez W.K., Hunt C.W., Guy M.A., Pritchard G.T., Swanson B.I., Treacher R.J., 1999. Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. J. Dairy Sci. 82, 611-617

Michalet-Doreau B., Fernandez I., Peyron C., Millet L., Fonty G., 2001. Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents. Reprod. Nutr. Develop. 41, 187-194

Morgavi D.P., Newbold C.J., Beever D.E., Wallace R.J., 2000. Stability and stabilization of potential feed additive enzymes in rumen fluid. Enzyme Microb. Technol. 26, 171-177

Morgavi D.P., Beauchemin K.A., Nsereko V.L., Rode L.M., McAllister T.A., Iwaasa A.D., Wang Y., Yang W.Z., 2001. Resistance of feed enzymes to proteolytic inactivation by rumen microorganisms and gastrointestinal proteases. J. Anim. Sci. 79, 1621-1630

Nakashima Y., Ørskov E.R., 1989. Rumen degradation of straw. 7. Effects of chemical pre-treatment and addition of propionic acid on degradation characteristics of botanical fractions of barley straw treated with a cellulase preparation. Anim. Prod. 48, 543-551

Nussio L.G., Huber J.T., Theurer C.B., Nussio C.B., Santos J., Tarazon M., Lima-Filho R.O., Riggs B., Lamoreaux M., Treacher R.J., 1997. Influence of a cellulase/xylanase complex (C/X) on lactational performance of dairy cows fed alfalfa hay (AH) based diet. J. Dairy Sci. 80, Suppl. 1, 220 (Abstr.)

Purkarthofer H., Steiner W., 1995. Induction of endo-beta-xylanase in the fungus *Thermomyces lanuginosus*. Enzyme Miocrob. Technol. 17, 114 -118

Schingoethe D.J., Stegeman G.A., Treacher R.J., 1999. Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. J. Dairy Sci. 82, 996-1003

Stokes M.R., 1992. Effects of an enzyme mixture, an inoculant and their interaction on silage fermentation and dairy production. J. Dairy Sci. 75, 764-773.

Wang Y., McAllister T.A., Rode L.M., Beauchemin K.A., Morgavi D.P., Nsereko V.L., Iwaasa A.D., Yang W., 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in Rumen Simulation Technique (Rusitec). Brit. J. Nutr. 85, 325-332

STRESZCZENIE

Aktywność i stabilność preparatu grzybowego 1,4-beta-endoksylanazy w żwaczu owiec

Doświadczenie przeprowadzono na 1-3 letnich skopach merynosach z przetokami żwacza celem określenia stabilności aktywności 1,4-beta-endoksylanazy z preparatu enzymatycznego, handlowo dostępnego (Rumino-Zyme, o aktywności 250 FXU/g, Dr Bata Ltd., Węgry). Do oznaczania aktywności ksylanazy w płynie żwacza i stałych próbach zastosowano technikę „Remazol blue", prostą, szybką i pewną.

Aktywność ksylanazy w żwaczu głodzonych skopów wynosiła 20-30 FXU/l, i była o około 2,5 razy większa w dwie i cztery godziny po karmieniu niż przed podaniem paszy. Podanie bezpośrednio do żwacza egzogenicznej ksylanazy zwiększało aktywność enzymatyczną. Po osiągnięciu piku aktywności w 5 minut po podaniu preparatu aktywność enzymatyczna zmniejszała się w czasie po jej podaniu do żwacza, z półokresem trwania 45-50 minut.

Wyniki przeprowadzonych badań nie potwierdzają wcześniejszych obserwacji o stałej aktywności zewnętrznych NSP-enzymów w warunkach *in vivo*.