THE ROLE OF BACTERIAL XYLANASES IN RUMINAL DIGESTION OF PLANT POLYSACCHARIDES

ULOGA BAKTERIJSKIH KSILANAZA U PROBAVLJANJU BILJNIH POLISAHARIDA U BURAGU

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SUMMARY

Among hemicelluloses of plant cell walls xylans constitute the major portion and represent an important substrate in ruminant feedstuff that could be degraded only via microbial xylanolytic enzymes.

Prevotella ruminicola B_14 is a strictly anaerobic, Gram-negative, polysacharide-degrading rumen bacterium. Three main xylanolytic acitivities were detected in this strain: endoxylanase, β-xylosidase and α-arabinofuranosidase. Xylanase activity was found to be inducible. Ten bacteriophage clones expressing xylanase activity were isolated from λ EMBL3 genomic DNA library of Prevotella ruminicola B_14. Clones represented four distincs chromosomal regions, based on restriction enzyme analysis. Three groups of clones encoded activity against oat spelt xylan. In clone 5 activities against pNP-arabinofuranoside and pNxyloside were found to be encoded separately from endoxylanase activity. The fourth region encoded activity against carboxymethylcellulose (CMC) and lichenan, in addition to xylan.

According to the results of oxygen, pH and temperature stability, native and cloned enzymes could be suitable for use as feed additives for monogastric animals. Genetically modified bacteria with enhanced fibrolytic activity could be used for introduction into the rumen.

INTRODUCTION

A review of recent trends in animal science shows that modern biotechnology has improved the nutritive value of feeds and forage. The improvement means mostly the production of a diversed range of feed grade enzymes - native ones or based on genetic manipulations. Molecular biology strategies, initially directed to enhancing growth, are now shifting in emphasis towards the transfer of genes for enhanced nutrient digestion.

At present several enzyme preparations are commercially used in animal nutrition. Phytases improve the digestability and availability of phosphorus in poultry and pig diets (Beers and Jongbloed, 1993). β-glucanase and xylanase activities added to barley and rye based diets for broilers improve weight gain and feed conversion efficiency while at

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the same time reduce problems of sticky droppings and wet litter (Petterson et al., 1990). In pig production, supplementation of the diet with a blend of amy-lase, proteinases and cellulases in the immediate post - weaning period, is considered to have potential in enhancing growth rate and preventing enteric disease (Close, 1992).

Cellulase and hemicellulase enzymes or microbial inoculants containing these enzymatic activities are used as silage additives to stimulate the release of sugars from poorer quality forage and thereby enhancing preservation (Forsberg et al., 1993).

With the development over the past few years of techniques for the introduction of genes into domestic animals (Briskin et al. 1991), there is the option of expressing glycanase genes in the animal itself rather than adding microbial enzymes or genetically manipulated microorganisms into the feed. To be beneficial the enzymes should be expressed in the appropriate tissue such as the pancreas for maximum effect.

The cloning and characterisation of genes coding for cellulases and hemicellulases of ruminal bacteria and fungi gives the opportunity to obtain genes coding for enzyme systems that have unique roles in ruminal fibre digestion. These genes can be used for the genetic enhancement of fibre digestion in domestic animals. More than 20 cellulase and xylanase genes have been cloned from ruminal bacteria and fungi and characterized, and many others are being analyzed. Practically all enzymes characterized in recent years are from the strongest cellulolytic or xylanolytic microorganisms and only a few, among them are xylanolytic enzymes from *Prevotella ruminicola* B_14, from other microorganisms.

Prevotella ruminicola represents a group of strictly anaerobic, Gram-negative bacteria that can account for a large portion of the total culturable bacteria in the rumen. Although they are not cellulolytic, *Prevotella ruminicola* strains related to B_14 an 23 produce abundant xylanase and endoglucanase activity and are thought to contributre to the degradation and utilisation of plant polysaccharides in the rumen. Genes encoding xylanase and endoglucanase activities have been previously isolated from several strains of *Prevotella ruminicola* , but the encoded enzymes have so far been found to be related to endoglucanases of family A, and no specific family F or G xylanases have been reported. We recently reported (Gasparič et al. 1995) that *Prevotella ruminicola* B_14 possesses at least three genes encoding relatively specific xylanases, one of which is adjacent to the region encoding β-xylosidase/α-L-arabinofuranosidase activity, in addition to a gene encoding an endoglucanase that has been previously isolated (Matsushita et al. 1991).

Although three main xylanolytic genes were succesfully isolated, neither cloned nor native xylanolytic enzymes were purified and biochemicaly characterised. It is possible that native enzymes possess slightly different biochemical and physiological characteristics than cloned enzyme products. We performed some classical microbiological and biochemical experiments with Prevotella ruminicola B_14 in order to prove the relevant role and meanining of its xylanolytic enzymes in the rumen.

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages

The rumen bacterium *Prevotella ruminicola* B_14, wild strain (J.B. Russell Cornell University, USA) was used for enzyme analysis, inducibility tests and DNA preparations. Recombinant λ EMBL3 bacteriophages were grown on E. coli P2392 (aP2 lysogen of LE392). E. coli HB101 and E. coli DH5α were used as the host strains for subclones made in plasmids pUC18 and pUC19.

Media and culture conditions

Prevotella ruminicola B_14 was grown anaerobicaly in M2 medium or in modified M2 media according to special purposes (Hobson 1969) at 38°C under 100% CO_2. Recombinant bacteriophages were plated and isolated from the BBL medium (10 g/l BBL trypticase, 5 g/l NaCl, 10 mM $MgSO_4$, with 6 g/l agarose (top layer) or 15 g/l agar (bottom layer)). For screening the xylanolytic

activity, oat spelt or birch wood xylane was added to the final concentration of 0,2% to the top layer. Recombinant *E. coli* strains were grown in LB medium containing 60 μl of ampicillin per ml.

Determinations of xylanolytic enzyme activities (native and cloned enzymes)

Quantitative determinations of endoxylanase activity were done by reducing sugar test according to the method of Lever (1977) and using oat spelt xylan as a substrate. α-L-arabinofuranosidase and β-xylosidase activities were determined by the method of Poutanen (1988) using p-nitrophenyl-α-L-arabinofuranoside and p-nitrophenyl-β-D-xyloside as substrate, respectively. Microbial cell proteins and the proteins of crude enzyme extracts were determined by the method of Lowry (1951).

Xylanolytic activity was also detected following SDS polyacrylamide gel electrophoresis (SDS PAGE) of Prevotella ruminicola B,4 cell proteins. SDS PAGE was done according to the procedure of Laemmli (1970). The procedure was modified by adding 0,2% of oat spelt xylan into the stacking gel (Saul et al., 1990). Xylanolytic enzymes were detected on the gel as clearing zones after staining the gel with alcalic solution of Congo red dye.

Induction and repression of xylanase activity in *Prevotella ruminicola* $B_1 4$

For induction studies, M2 medium was modified to contain 0,6% oat spelt xylan (M2-XYL medium) or 0.6% glucose (M2-GLU medium) as the sole source of energy. *Prevotella ruminicola* cells were grown in 700 ml of modified M2 medium for 60 hours at 38°C and samples (10 ml) taken at different times of incubation anaerobically. Cells were centrifuged (20 min, 3000 x g, 4°C), washed twice with 50 mM Na-phosphate buffer pH 6.5, frozen at -20°C and disrupted by osmotic shock before assaying xylanase and CM-cellulase activities. For testing the enzyme repression with monosaccharides Prevotella ruminicola B,4 was cultivated in Hungate tubes in the following media: M2-XYL medium, M2-XYL medium with 0,6% arabinose, M2-XYL medium with 0,6% xylose and M2-XYL medium with 0,6% glucose. Xylanolytic activities of cultures grown with monosaccharides were compared after 20 hours incubation to the xylanolytic activity of *Prevotella ruminicola* $B_1 4$ grown on xylan as the sole carbon source by reducing sugar test. At the same time *Prevotella ruminicola* B,4 was grown in M2 media containing only 0,6% of monosaccharides and the xylanolytic activities were compared to those on xylan.

pH, temperature and oxygen sensitivity of native and cloned xylanolytic enzymes

The influence of pH on native endoxylanase activity was tested by incubating the osmotically shocked and ultrasonicated cells of *Prevotella ruminicola* $B_1 4$ 120 minutes with 1% oat spelt xylan diluted in buffers of different pH values from 1,0 to 8,0. Reducing sugars were assayed and specific activities calculated. pH stability of endoxylanase at extremely acidic pHs was determined as follows: the cells were preincubated for 30 minutes in buffers at pH values 1,0, 1,5, 2,0 and 6,0 at 37°C. The retained enzymatic activity was determined after neutralisation of tet mixtures and further incubation at pH=6 and 37°C for 120 minutes. Reducing sugars were assayed and the percentage of the retained activity was calculated. The enzymatic activity detected after preincubation at pH=6 was taken as 100% activity.

Oxygen sensitivity of recombinant cells of *E. coli* was assayed as follows: cells were desintegrated with ultrasonification. pNP-xyloside in 50 mM Na-phosphate buffer pH 6,5 was used as a substrate. Recombinant xylanolytic enzymes were analyzed for their activity in aerated buffer without DTT (2 mM) and with DTT (2 mM) and in anaerobic buffer with and without DTT for 15 minutes at 37°C.

For termostability test recombinant *E. coli* cells were desintegrated with ultrasonification. Cell extracts were preincubated for 0,5, 10, 20 and 30 minutes at 50°C. These extracts were incubated with two different substrates: birchwood xylan and oat spelt xylan (1%) in phosphate buffer pH 6,5 with DTT (2 mM) am 37°C for 30 minutes.

Library construction and subcloning

All molecular genetic manipulations were prepared as written in standard procedures (Sambrok et al. 1989). Chromosomal DNA was extracted from *Prevotella ruminicola* as previously described (Avguštin et al. 1994) except that CsCl step was omitted, due to the phenol/chloroform treatment and elimination of RNA with RNase. A genomic library was constructed by ligating 9-23 kb fragments derived from a Sau3A partial digest of *Prevotella ruminicola* DNA with λ EMBL3 bacteriophage BamHI digested arms, followed by packaging in vitro with Gigapack II gold packaging extract (Stratagene). Bacteriophage DNA was isolated and subclones were obtained by digesting phage DNA with SalI and partially with EcoRI, followed by ligation with SalI or SalI/EcoRI cut pUC18 and transformation of *E. coli* HB101.

RESULTS AND DISCUSION

Xylanolytic activites of *Prevotella ruminicola* B_14

SDS PAGE xylanograms of *Prevotella ruminicola* B_14 cell proteins showed two clear endoxylanolytic bands at 29 kDa and 66 kDa and two additional but more faint bands at 26 kDa and 89 kDa. We can claim that we proved multiple xylanolytic enzymes of Prevotella ruminicola B_14. Maximal xylanolytic activity determined according to reducing sugars test was 340,67 nM RS/ mg PROT/ min, maximal β-xylosidase activity was 22,11 nM XYL/mg PROT/ min and maximal α-arabinofuranosidase activity was 73,7 nM ARA/ mg PROT/ min.

Induction and repression of xylanase activity in *Prevotella ruminicola* B_14

In *Prevotella ruminicola* B_14, xylanase activity was found to be increased between 20-and 40-fold after 12 h growth with xylan as energy source, compared to growth on glucose. CM-cellulase activity was also higher in xylan grown cells, but by a smaller factor between 2- and 7-fold. A strong induction of xylanolytic activity is also seen from the comparison of enzyme activities of cultures grown on xylan and monosaccharides as sole carbon sources (table 1). In addition the induction of xylanolytic activity is clearly shown on SDS PAGE xylanogram. The repression (possibly the catabolite repression) of xylanolytic activity with monosaccharides, that are potential degradation products of xylan, is shown in table 2.

Table 1. Xylanolytic activities of *Prevotella ruminicola* B_14 grown on different monosaccharides and xylan

Tablica 1. Ksilanolitične aktivnosti *Prevotella ruminicola* B_14 uzgajanje na raznim monosaharidima i ksilanu

MEDIUM	Xylanolytic activity (nM RS/ mg PROT/min)	Ratios to the activity on glucose
M2-GLUCOSE	24.59	-
M2-ARABINOSE	30.59	1.3 : 1
M2-XYLOSE	54.08	2.2 : 1
M2-XYLAN	340.67	13.8 : 1

Table 2. Repression of *Prevotella ruminicola* B_14 xylanolytic activity with monosaccharides

Tablica 2. Potiskivanje ksilanolitične aktivnosti *Prevotella ruminicola* B_14 monosaharidima

MEDIUM	Xylanolytic activity (nM RS/ mg PROT/ min)	Ratios to the activity on xylan
M2-XYL	233.15	-
M2-XYL + 0.6% arabinose	66.12	0.28 : 1
M2-XYL + 0.6% xylose	104.38	0.45 : 1
M2-XYL + 0.6% glucose	57.00	0.24 : 1

Isolation of xylanase genes from *Prevotella ruminicola* B_14

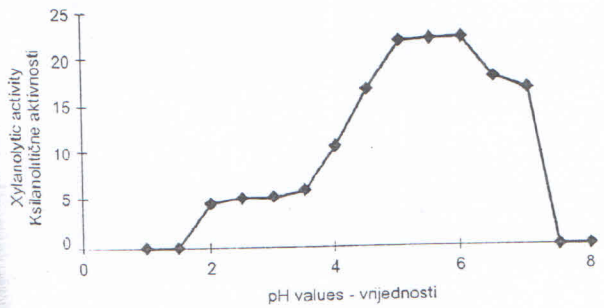
We isolated recombinant λ EMBL3 bacteriophages form ten xylanase-positive plaques obtained from aproximately 3000 plaques screened, from a genomic library of *Prevotella ruminicola* B_14. Xylanase positive phages were classified into four groups due to the restriction analysis results and one representative of each group was chosen for

further investigation. With cross hybridization of all four recombinant bacteriophages we confirmed that each phage carried non-homologous, non-overlapping regions of the *Prevotella ruminicola* B_14 chromosome (Gasparič et al. 1995).

Activities encoded by phage clones, 3, 5, 6 and 8: A 6,8 kb EcoRI fragment from the bacteriophage clone was subcloned in pUC18 and expressed enzymatic activities against oat spelt xylan, birchwood xylan, pNP-xyloside (pNPX) and pNP-arabinofuranoside (pNPA) (Fig 1). Subclones obtained from phage 3 expressing xylanase activity were isolated due to the formation of the clearing zones in an oat spelt xylan agarose overlays. They carried a 4,7-kb EcoRI fragment (Fig. 1). We could not obtain xylanase positive clones from phage 6. Xylanase was the only significant activity detected in lysates from phages 3 and 6. Bacteriophage 8 or its plasmid subclone 8/19 carrying a 6-kb EcoRI expressed activity against CM-cellulose (carboxymethyl cellulose) and the β(1,3-1,4)-glucans lichenan and barley β-glucan in addition to xylanase activity. Restriction enzyme analysis indicated that recombinant bacteriophage clone 8 corresponds with a region isolated from the same strain by Matsushita et al. (1989).

pH, temperature and oxygen sensitivity of native and cloned xylanolytic enzymes

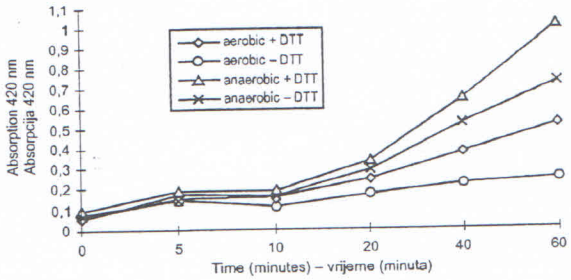
Native xylanase activity was extremely low in a pH range from 1 to 3,5 and exhibited maximal activity in a pH range from 4,5 to 6,5 (graph 1). The important fact is that 75% of the xylanase activity was recovered after preincubation at pH=1 and 92% at pH=2.



Graph 1: The influence of pH on native xylanolytic activity

Grafikon 1: Utjecaj pH na nativnu ksilanolitičnu aktivnost

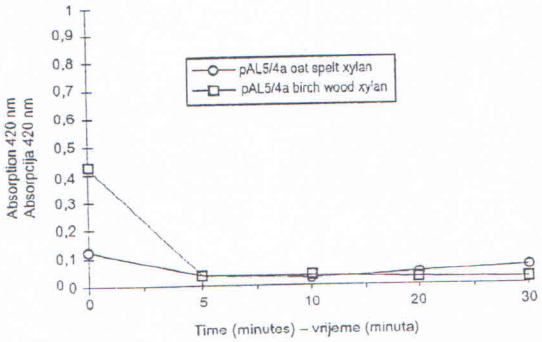
Although xylanases and cellulases of anaerobic microorganisms are usually not inactivated with oxygenation there are some exceptions. This was the reason for testing our recombinant xylanolytic enzymes for oxygen sensitivity. The only significant oxygen inhibition was shown for an enzyme arabinofuranosidase/β-xylosidase after incubation of 15 minutes at 37°C. This enzyme in encoded by a DNA fragment of the plasmid clone pAL5/4a (Graph 2.).



Graph 2: Thermostability of arabinosidase/β-xylosidase.

Grafikon 2: Termostabilnost arabinosidaze/β-ksilosidaze

Since cloned enzymes could response to the incubation conditions differently from the native enzymes we tested thermostability of the most interesting plasmide subclone encoding endoxylanase and arabinosidase/β-xylosidase activities, clone pAL5/4a. The test shows an obvious temperature inactivation after incubation at 50°C for 5 minutes (Graph 3.).



Graph 3: Temperature inactivation of enzymes endoxylanase and arabinosidase/β-xylosidase followed by incubation on oat spelt xylan and on birchwood xylan

Grafikon 3: Onesposobljavanje temperaturom enzima endoksilanaze i arabinodaze/β-ksilosidaze prije inkubacije na ksilanu zobi i ksilanu brezovine.

CONCLUSIONS

Our biochemical and molecular biology experiments revealed three main xylanolytic enzyme activities in rumen bacterium Prevotella ruminicola B_14: multiple endoxylanase activity, β-xylosidase activita and α-arabinofuranosidase activity: These enzymes are able to achieve the complete degradation of polymeric xylane, which is the main hemicellulose in ruminant feedstuff. Endoxylanase splits xylan chains to xylooligosaccharides, xylosidase releases xylose residues by its exo-action and arabinofuranosidase is an important debranching enzyme cleaving the bondages between the main xylan chain and its branches. Comparison at a molecular level of the xylanolytic system of a noncelluloytic rumen species such as Prevotella ruminicola with that of cellulolytic rumen species or succesfull isolation and biochemical characterisation of enzymes should provide important insights into roles of individual gene products-enzymes in plant cell wall breakdown.

We are able to develop genetically modified bacteria with enhanced fibrolytic activity for introduction into the rumen. These same genes can be introduced into monogastric animals to enhance their capacity to digest complex carbohydrates. The primary problems to be solved concerning construction of modified bacteria and transgenic animals are development of appropriate vectors, antibiotic resistance markers (bacteria), and promoters for heterologous genes. Features such as nature of glucanases and xylanases, their catalytic properties and stability/resistance against proteolytic degradation in the rumen and in the small intestine of monogastric animals are particularly important.

If we consider the potential biotechnological use of our xylanolytic enzymes we could claim that the native endoxylanase of Prevotella ruminicola B_14 could not be very active in acidic conditions of the stomach of monogastric animals, but it could be active in the crop of poultry and the most important fact is that it could survive the extremely acidic conditions of stomach and could be efficiently active in the small intestine.

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SAŽETAK

Među hemicelulozama stijenka biljnih stanica ksilani tvore glavni dio i predstavljaju u hrani preživača važni supstrat, koji se može razgraditi samo mikrobijskim ksilanolitičnim enzimima.

Prevotella ruminicola B_14 je strogo anerobična gram pozitivna bakterija buraga što razgrađuje polisaharide. Tri glavne ksilanolitične aktivnosti otkrivene u ovom soju su: endoksilanaza, β-ksilosidaza i α-arabinofuranosidaza. Utvrđeno je da se aktivnost ksilanaze može izazvati iz λEMBL3 genomske grupe DNA B_14 Prevotella ruminicola, izolirane iz 10 bakteriofagnih klona koji očituju aktivnost ksilanaze. Kloni predstavljaju 4 izrazita kromosomska područja na osnovi analize ograničenja enzima. Tri skupine klona sadrže kodiranu aktivnost protiv ksilana zobi (oat spelt). U klonu 5 utvrđeno je da su aktivnosti protiv pNP-arabinofuranozida i pNksilosida kodirane odvojeno od aktivnosti endoksilanaze. Osim ksilana četvrto područje sadrži kodiranu aktivnost protiv karboksimetilceluloze i lichenana.

Prema rezultatima, stabilnosti kisika pH i temperature, nativni (urođeni) i klonirani enzimi mogli bi se upotrijebiti kao dodaci hrani mnogih životinja. Genetički modificirane bakterije s povišenom fibrolitičnom aktivnošću mogle bi se upotrijebiti za uvođenje u burag.