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XYLANOLYTIC ENZYME SYSTEM OF RUMEN BACTERIUM *Prevotella bryantii* $B_1$4

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A B S T R A C T

Prevotella spp. are recognised as one of the most numerous strictly anaerobic bacteria inhabiting the rumen. Potentially significant activities include the degradation of plant cell wall polysaccharides, starch, proteins and peptides. *P. bryantii* $B_1$4 is not cellulolytic but actively degrades hemicellulose xylan and carries multiple xylanase genes. Four regions encoding xylanase activity have been isolated, one of which encodes a previously isolated CMC-ase. Of the remaining regions, one encodes activities against *p*-nitrophenyl-β-xyloside and *p*-nitrophenyl-α-L-arabinofuranoside (genes *xyn* A and *xyn*B). The gene *xyn*C encodes another endoxylanase. SDS PAGE xylanograms revealed four endoxylanolytic bands at 29 kDa, 45 kDa, 66 kDa and 88 kDa. The majority of endoxylanase and CMC-ase activity was found in periplasmic cell fraction while most of the α-L-arabinofuranosidase and β-xylosidase activities were found in the crude membrane fraction. HPLC separation of periplasmic proteins by CIM DEAE 8 tubes resulted in partial isolation of CMC-ase and 66-kDa endoxylanase.

Key words: microbiology / bacteria / *Prevotella bryantii* / enzymes / xylanases / rumen

KSILANOLITIČNI ENCIMSKI SISTEM VAMPNE BAKTERIJE *Prevotella bryantii* $B_1$4

I Z V L E Č E K

Vrste rodu *Prevotella* so med najpogostejšimi striktno anaerobnimi bakterijskimi vrstami v vampu. Njihovi pomembni učinki vključujejo razgradnjo polisaharidov rastlinskih celičnih sten, razgradnjo škroba, proteinov in peptidov. *P. bryantii* $B_1$4 je necelulolitična vrsta, vendar zelo aktivno razgrajuje hemicelulozo ksilan in ima multiple ksilanazne gene. Izolirane so bile štiri regije DNK, ki kodirajo ksilanolitične aktivnosti, od katerih ena ustreza širokospecifični endoglukanazi. Od ostalih treh regij ena kodira aktivnost proti *p*-nitrofenil-β-ksilozidu in *p*-nitrofenil-α-L-arabinofuranozidu poleg endoksilanazne aktivnosti (gena *xyn* A in *xyn*B). Gen *xyn*C kodira drugo endoksilanazo. Ksilanolitična aktivnost *P. bryantii* $B_1$4 je močno inducibilna. SDS PAGE ksilanogrami pokažejo štiri endoksilanolitična območja: pri 29 kDa, 45 kDa, 66 kDa in 88 kDa. Po celični frakcionaciji smo večino endoksilanazne in CMC-azne aktivnosti našli v periplazmatski celični frakciji, večino β-ksilozidazne in α-L-arabinofuranozidazne aktivnosti pa v membranski frakciji. S pomočjo CIM DEAE 8 cevnih modulov sta bili s HPLC tehniko delno izolirali CMC-aza in 66-kDa endoksilanazo iz periplazmatske frakcije.

Ključne besede: mikrobiologija / bakterije / *Prevotella bryantii* / encimi / ksilanaze / vamp

INTRODUCTION

Xylan is beside cellulose one of the major plant structural polysaccharides and represents an important energy source in ruminant feedstocks. Isolated β-1,4-xylans belong to heteropolymerous hemicelluloses. They are substituded and branched to different degrees. Xylan backbone is composed of β-1,4-linked D-xylopyranosyl residues carrying the following substituents: α-2,3-linked L-arabinose, α-1,2-linked glucuronic or 4-*O*-methyl-D-glucuronic acid and via ester bonds linked ferulic, p-coumaric or acetic acid.

Herbivorous animals are completely dependent on their microbial symbionts in exploiting this energy source. Microbes possess a whole battery of xylanolytic enzymes due to the complex structure of xylan: the polyxylopyranosyl backbone is hydrolised by endo-β-1,4-xylanases and β-xylosidases, the side groups and chains are cut off by α-L-arabinofuranosidases, 4-*O*-methyl-D-glucuronidases, acetylxylan esterases and esterases of ferulic and *p*-coumaric acid (Malburg *et al*., 1992). The most active rumen xylanolytic bacterial species belong to *Butyrivibrio*, *Fibrobacter*, *Ruminococcus*, *Bacteroides* and *Prevotella*. Their cellulolytic and xylanolytic enzymes probably act sequentially and synergistically in substrate breakdown and they contribute to a complex microbial cross-feeding system in the rumen.

Prevotella spp. formerly classified as *Bacteroides ruminicola* are among the most numerous organisms isolated from the rumens of cattle and sheep (van Gylswyk, 1990) and they are generally thought to make a significant contribution to the degradation of starch, proteins and cell wall substances (Stewart *et al*., 1997). They are strictly anaerobic, Gram-negative pleiomorfic rods. Their important enzymatic activities are: degradation of proteins and peptides, hydrolysis of starch, pectin and xylan, but they do not hydrolyse native cellulose. Among 23 tested species of genus *Prevotella* the highest xylanolytic activity was detected in *P. bryantii* $B_1$4. This was the reason to examine its xylanolytic enzyme system more precisely. Purified or partially purified xylanases also have a broad potential of beeing used as feed additives in ruminant and monogastric animal nutrition and have a potential of some other biotechnological applications.

MATERIAL AND METHODS

The rumen bacterium *Prevotella bryantii* $B_1$4 was grown in anaerobic conditions at 38^{o}C in M2-medium under 100 % CO_2 (Hobson, 1969). For induction studies *P. bryantii* $B_1$4 was grown in M2X-medium, where the usual carbon sources were replaced by 0,6 % oat spelt xylan. Recombinant bacteriophages λEMBL3 were cultivated in BBL medium on *E. coli* P2392 and Y1090. A genomic library was constructed by ligating 9 to 23 kb fragments derived from a Sau3A partial digest of *P. bryantii* $B_1$4 genomic DNA with λEMBL3 bacteriophage arms digested with Bam HI. In vitro packing of bacteriophage DNA using Gigapack gold II packing extract (Stratagene) followed. Subclones were prepared by cutting of bacteriophage DNA with the restriction enzymes SalI in EcoRI, subcloning into plasmids pUC18 and pUC19 and transformation of *E.coli* HB101. Enzymatic activities and characteristics of cloned products were analysed. Clone 5/4 was analysed more exactly and its nucleotide sequence determined. Xylanolytic activity was determined spectrophotometrically with 1% oat spelt xylan being used as substrate. Reducing sugars were detected according to the method of Lever (Lever, 1977). α-L-arabinofuranosidase and β-xylosidase activities were determined using appropriate synthetic p-nitrophenyl substrates. The liberated p-nitrophenyl was measured spectrophotometrically at 410 nm (Poutanen, 1988). SDS PAGE xylanograms of cloned gene products and sonicated cells

of *P. bryantii* $B_1$4 were prepared according to the method of Laemmli (1970), xylanolytically active enzymes were detected as clearing zones following the Congo red staining. Isoelectric focusing of *P. bryantii* $B_1$4 cell proteins was done on Multiphor II apparatus (Pharmacia). Sandwich gel techniques with xylan and carboxymethyl cellulose (CMC) were used for detection of xylanases. The osmotic shock release procedure was applied to study the localization of xylanolytic enzymes. Xylanolytic activities were determined for all cell fractions and the extracellular fraction. Periplasmic proteins were further separated on CIM DEAE tubes using HPLC techniques to partially isolate the 66 kDa endoxylanase and 88 KDa CMC-ase with xylanolytic activity (Štrancar *et al*., 1997).

RESULTS AND DISCUSION

Xylanolytic and CMC-ase activities in *P. bryantii* $B_1$4 were found to be inducible with xylan, the activities being 20 and 7 times higher respectively when grown on xylan as compared with glucose. SDS PAGE xylanograms showed two strong endoxylanolyticaly active proteins at 29 and 66 kDa when oat spelt xylan was incorporated into the gel. Additional clearing zones were detected at 45 and 82-88 kDa, when the soluble fraction of oat spelt xylan was used and additional reducing agents applied. *P. bryantii* $B_1$4 possesses at least four different proteins with endoxylanolytic activity, of which the 82 and 88 kDa proteins belong to the formerly described and cloned CMC-ase (Matsushita *et al*., 1990). Beside its CMC-ase activity endoxylanolytic activity was clearly proved for the first time.

Ten bacteriophage clones expressing xylanolytic activity were isolated from λEMBL3 genomic DNA library of *P. bryantii* $B_1$4. Following the restriction analysis and DNA hybridisation results, xylanase positive phage clones were classified into 4 groups representing four distinct chromosomal regions. The gene *xynC* (represented by the clone 3/24) coded for a 66 kDa enzyme with endoxylanolytic activity. The products of the two linked genes *xyn A* and *xynB* (represented by the clone 5/4a, MW = 29 kDa) expressed endoxylanolytic activity beside oxigen sensitive β-xylosidase and α-L-arabinofuranosidase activity. Because of the biotechnological potential this clone was patented. The clone 8/19 encoded a broad specificity endoxylanase expressing as 82 and 88 kDa proteins. Following isoelectric focusing the isoelectric points pI_1 = 5.0 and pI_2 = 5.1 were determined. The clone λ6 was not analysed further because of its low expressed activity.

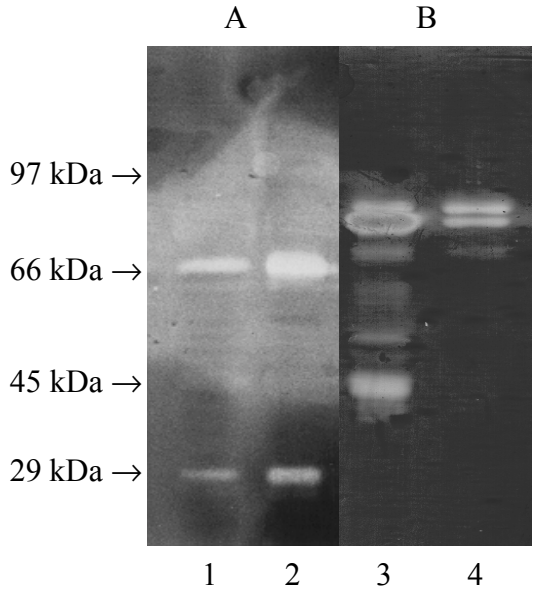
P. bryantii $B_1$4 possesses multiple xylanases that differ in their cell locations. Less than 25 % of total xylanase and CMC-ase activity belongs to the extracellular fraction. A significant portion of β-xylosidase activity appears to be membrane associated. The majority of endoxylanase and CMC-ase activity was found in periplasmic cell fraction and it seems likely that several of the multiple xylanases must be located there. It has been shown previously for colonic *Bacteroides* sp. that the bulk of xylanase activity is within the cell or periplasm (Salyers *et al*., 1995) and the same conclusion has also been suggested for *P. rumnicola* (Daniel *et al*., 1995). The proteins correspondent for these two activities were partially isolated from nonconcentrated periplasm using HPLC techniques and BIA CIM DEAE tubes (see figure 2). Enzymes in periplasmic space are partially protected against different proteinases and a strongly competitive rumen ecosystem in general. The xylanolytic enzymes of *P. bryantii* $B_1$4 probably act sequentially and cooperate with xylanases and cellulases of other microbial species in the rumen in the degradation of the substrate. *Prevotella* spp. as a group are thought to utilise soluble xylan molecules released by the activities of cellulolytic species in the rumen (Dehority, 1979).

Table 1. Specific enzymatic activities and proportions of the total activities in *P. bryantii* $B_1$4 cell fractions

Preglednica 1. Specifične encimske aktivnosti in deleži skupne aktivnosti v celičnih frakcijah *P. bryantii* $B_1$4

CELL FRACTION Celična frakcija	XYLANOLYT. ACTIVITY Ksilanolitična aktivnost	CMC-ASE ACTIVITY CMC-azna aktivnost	ARABINOFURANO-SIDASE ACTIVITY Arabinofuranozid. aktivnost	β-XYLOASIDASE ACTIVITY β-ksilozidazna aktivnost
Periplasm Periplazma	526,48 **(62 %)**	476,26 **(99,5 %)**	0 **(0)**	0 **(0)**
Soluble fraction Topna frakcija	18,39 **(4,5 %)**	1,02 **(0,5 %)**	5,65 **(24,3 %)**	1,46 **(12, 9 %)**
Membrane fraction Membranska frakcija	190,24 **(33,4 %)**	0 **(0)**	24,97 **(75,7 %)**	13,87 **(87,1 %)**

The first values in the table are specific enzyme activities expressed in nM of product per mg protein per minute. The values in parentheses are the percentages of the total cell - associated activity in each fraction.
Prve vrednosti v preglednici so specifične encimske aktivnosti izražene v nM produkta na mg proteinov na minuto. Vrednosti v oklepajih so odstotki skupne na celico vezane aktivnosti v vsaki frakciji.



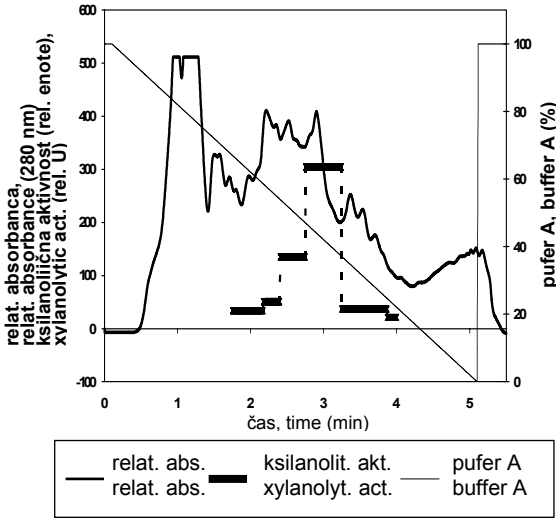


Figure 1 (left). Multiple xylanases of *P. bryantii* $B_1$4 are seen as clearing zones. A-native oat spelt xylan, B-soluble oat spelt xylan, 1-cells grown in M2, 2, 3-cells grown in M2X, 4- a sample of periplasm

Slika 1 (levo). Multiplost ksilanaz pri *P. bryantii* $B_1$4-ksilanaze so vidne kot prosojne proge. A-naravni ksilan ovsenih plev, B-topni ksilan ovsenih plev; 1-celice gojene v M2, 2, 3,-celice gojene v M2X, 4-vzorec periplazme

Figure 2 (right). HPLC fractionation of periplasmic proteins on CIM DEAE tubes. The proteins of the fourth fraction were loaded again onto the same tube and eluted under the same conditions to get separated 66 KDa endoxylanase and CMC-ase

Slika 2 (desno). HPLC ločitev periplazmatskih proteinov s CIM DEAE cevnimi moduli. Proteine četrte frakcije smo ponovno nanesli na isti cevni modul in jih eluirali pri istih pogojih. Tako smo dobili ločeni 66 KDa endoksilanazo in CMC-azo.

POVZETEK

Prežvekovalci so pri presnovi rastlinskih snovi odvisni od celulolitičnih in hemicelulolitičnih mikroorganizmov. Vrste iz rodu *Prevotella* so med najštevilnejšimi iz vampa govedi in ovc izoliranih mikroorganizmov. Številni sevi vampnih *Prevotell* proizvajajo hemicelulaze, pektinaze in endoglukanaze in tako sodelujejo s celulolitičnimi mikroorganizmi. *P. bryantii* $B_1$4 je bila opisana kot močno ksilanolitična bakterija. Najmanj štiri ksilanolitične encime je možno zaslediti na SDS-PAGE ksilanogramih in najmanj štiri kromosomalne regije nosijo zapise za ksilanazno aktivnost. Najzanimivejše odkritje je periplazmatska lokacija endoksilanazne in CMC-azne aktivnosti. Aktivne beljakovine smo uspešno delno očistili iz nekoncentrirane periplazme s HPLC. S frakcijo, ki je vsebovala ksilanolitično najbolj aktiven 66-kDa protein, smo imunizirali miši za pridobivanje monoklonskih protiteles.

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