ISOLATION OF ENDOXYLANASES FROM ANAEROBIC BACTERIUM *butyrivibrio* SP. STRAIN MZ5 IS POSSIBLE BY ANION EXCHANGE CHROMATOGRAPHY ON CIM® DEAE-8 MONOLYTHIC COLUMN

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Abstract

The xylanolytically active enzymes are of great interest for the industry and as feed additives as well. Therefore an effort was made to search for the microbial strains capable to degrade xylan. One of the most active rumen bacteria was *Butyrivibrio* sp. strain Mz5 possessing multiple xylanolytic enzyme system. In the present work the procedure for the separation of two of them is outlined. This was successfully done by anion exchange chromatography followed by gel filtration. Using the CIM® DEAE tube was the key isolating point that prevented otherwise frequent aggregation of the proteins and speeded up the procedure. The isolated enzymes were of 51 and 58 kDa.

Introduction

Ruminants are not capable to metabolize plant cell wall polysaccharides in forage by their endogenous enzymes. Therefore symbiotic microorganisms in the rumen play an important role in degradation of fiber, which is the major constituent of ruminants feed. The most important microbial enzymes involved in this process are cellulases and hemicellulases, among the later xylanases are most extensively studied. There are several enzymes that cleave xylan which represents the second major polysaccharide in plant cell walls.[1] Some of them attack the backbone of β (1-4) linked D-xyloses and are called endoxylanases (endo-1,4-β-D-xylanases, EC 3.2.1.8), the others remove substituents from complex xylan molecule (so called debranching enzymes). Endoxylanases possess a very high biotechnological potential. Isolated enzymes are widely used in pulp and paper industry for biobleaching, consequently the contamination of environment by inorganic bleachers is decreased significantly.[2] They are used as feed additives for better conversion of forage and reduction of health problems of monogastric animals, too.[3] Products of xylan hydrolysis promote beneficial growth of some probiotic bacteria like bifidobacteria and lactic acid bacteria in large

intestinum.[4] In food industry endoxylanases are applied for clarification of fruit juices and as texture modulators of dough.[5] Therefore it is of great interest to seek for new sources of xylanolytic enzymes with favorable properties – high temperature resistance, broad pH tolerance and good stability in extreme conditions. Unfortunately very few xylanolytic enzymes have been isolated and identified so far. This has been usually done after cloning the xylanase gene into host organism, usually accompanied by low expression levels.[6] Therefore each success in this field is of great importance.

Experimental

Bacterial strain, media and culture conditions: Butyrivibrio sp. strain Mz5 (ATCC BAA-455) was isolated in our lab in 1997 from the rumen of Holstein-Friesian cow with oat spelts xylan as the main carbon source in the growth medium.[7] Bacteria were grown for 24 hours at 37 °C under anaerobic conditions in defined medium without rumen fluid (DSMZ Medium 330)[8] where glucose, maltose, cellobiose, soluble starch and glycerol were replaced with oat spelts xylan in final concentration 0,5 %.

Preparation of cell extract: 1600 ml of cell culture was centrifuged at 4000 x g. The pellet was washed twice in sodium phosphate buffer (50 mM, pH = 6.5) and frozen in 160 ml of distilled water to achieve osmotic shock. Further on sonication was used for better cell disruption. Cell contents were separated from cell debris by centrifugation at 25.000 x g.

Purification of endoxylanases: The cell extract was applied onto a weak ion exchange monolithic column CIM® DEAE-8 (BIA Separations, Ljubljana, Slovenia) by Gradifrac MPLC system (Pharmacia-LKB). The column was equilibrated with 50 mM TRIS-Cl buffer (pH = 7.2) and proteins eluted by applying an increasing linear gradient from 0 to 1 M NaCl in equilibration buffer. The separation was done in 25 minutes with flow rate of 8 ml per minute. Fractions of 6 ml were collected. Enzymatically active fractions were pooled and concentrated by ultrafiltration using the 10 kDa cut-off ultrafilters (Amicon, Lexington, USA). Further separation of endoxylanases was performed by gel filtration on Sephacryl HR-200 column (1.6 x 92 cm) with 0.1 M NaCl in 20 mM TRIS-Cl buffer (pH = 7.2). The flow rate was 1 ml per minute.

Determination of specific xylanolytic activities: Concentrations of reducing

sugars were determined spectrophotometrically following 150 minutes incubation of samples with the substrate (1.0 % oat spelts xylan in 50 mM sodium phosphate buffer, pH = 6.5) at 37 °C according to the method of Lever.[9] Protein amounts were determined using the method of Lowry.[10] Specific enzymatic activities were expressed as nkat (nmols of products per second) per mg protein.

SDS-PAGE xylanograms: Sample proteins were denatured for 10 minutes at 80 °C and then separated by SDS polyacrylamide gel electrophoresis.[11] The procedure differed from the original one by the addition of 0.2 % oat spelts xylan into separating gels.[12] Proteins with endoxylanolytic activity were detected as clearing zones after renaturation, incubation and staining of the gel with alkaline solution of Congo red stain (Sigma). Enzyme molecular weights were determined according to the calibration curve with protein markers (Sigma, SDS-6H) on Coomassie blue stained parts of the gels.

Results and discussion

Rumen bacteria are of great importance for ruminants, which are incapable of degradation of fiber material that represents major part of their plant forage. The microbial symbionts degrade the polysaccharides into smaller molecules. Only those can be metabolized by the animals. Xylan is one of the important components of the plant cell wall. There are various genera of rumen bacteria that are capable to degrade xylan. The highest activity so far was detected for the strain Mz5 which was proved to belong to genus *Butyrivibrio*.[13] This can be due to many xylanolytically active enzymes present in this bacterium. Up to 14 clearing zones on the xylanogram were detected, each corresponding the individual xylanase. Their molecular weights ranged from 26,7 to 145 kDa (Figure 1, lane C).

Individual xylanases were tried to be isolated by anion exchange chromatography. 160 ml of non-concentrated cell extract was applied to CIM® DEAE-8 ml monolithic column and 22 fractions were collected and tested for enzymatic activity. The highest enzymatic activity was detected in the fractions 4, 5, 12, 13 and 19 (Graph 1).

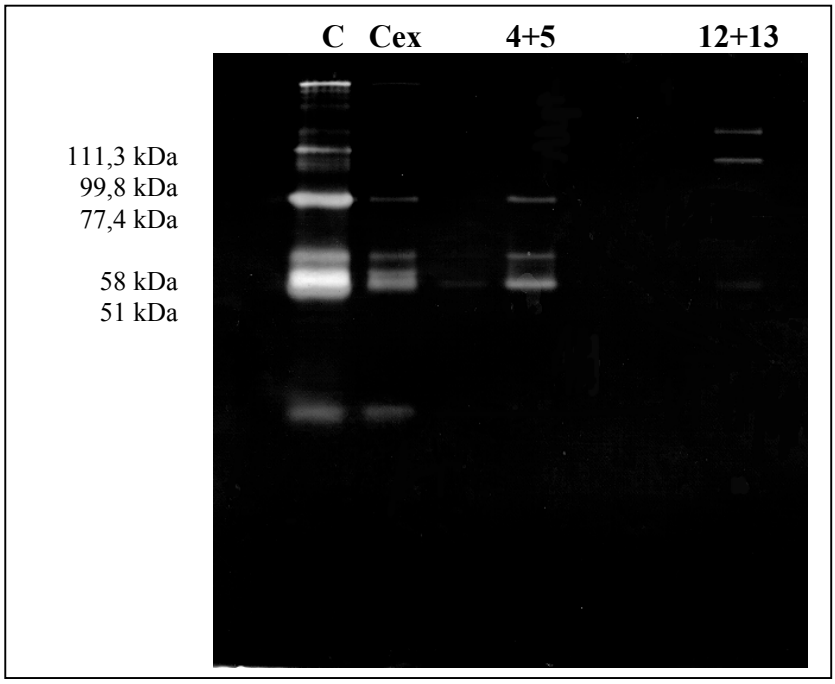
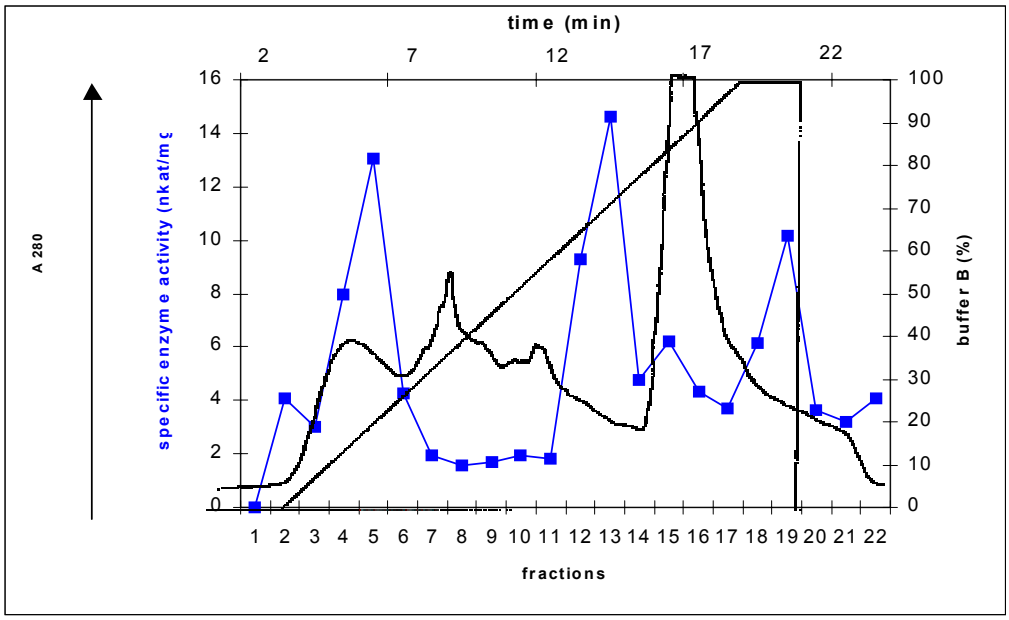


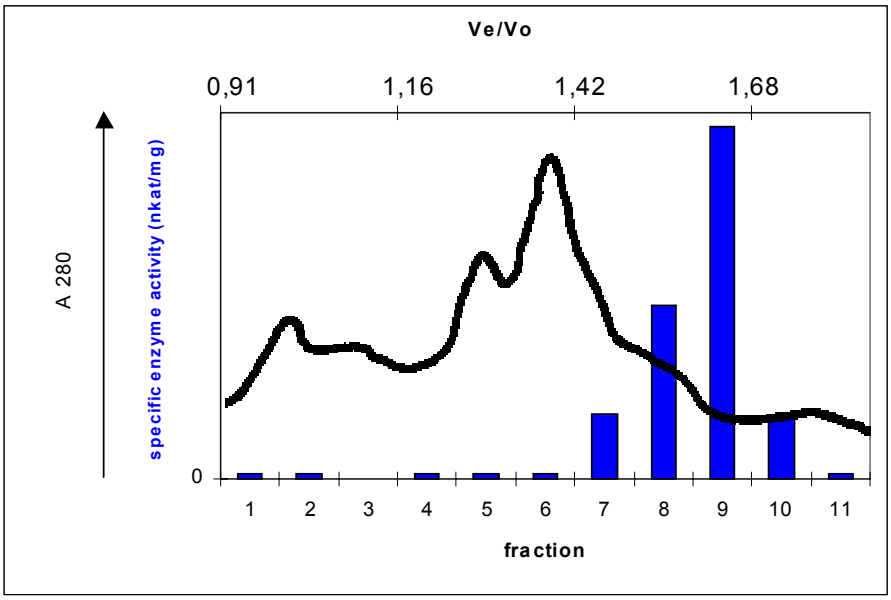
Figure 1: Xylanogram of the cell bound xylanases (C), xylanases from the cell extract (Cex) and the two enzymatically active peaks (4+5, 12+13) from anion exchange chromatography

The fractions 4 and 5 were joined together and 12 and 13 together. Joined fractions (4+5 and 12+13) were subjected to further separation and analysis. After revealing the xylanogram each sample revealed three clearing zones. They corresponded to the 77,4 kDa, 58 kDa and 51 kDa endoxylanases from the sample 4+5 and to the 111,3 kDa, 99,8 kDa and 51 kDa enzyme from the sample 12+13 (Figure 1, lanes 4+5 and 12+13 respectively).

The size exclusion method was used for further separation of the individual enzymes. Collected fractions were tested for the xylanase activity again. The fractions 7, 8 and 9 of the sample 4+5 turned out to posses the highest activity. The presence of xylanases was checked on xylanograms and each fraction was proved to contain one individual endoxylanase (Graph 2 and figure 2, lanes 7 to 9).

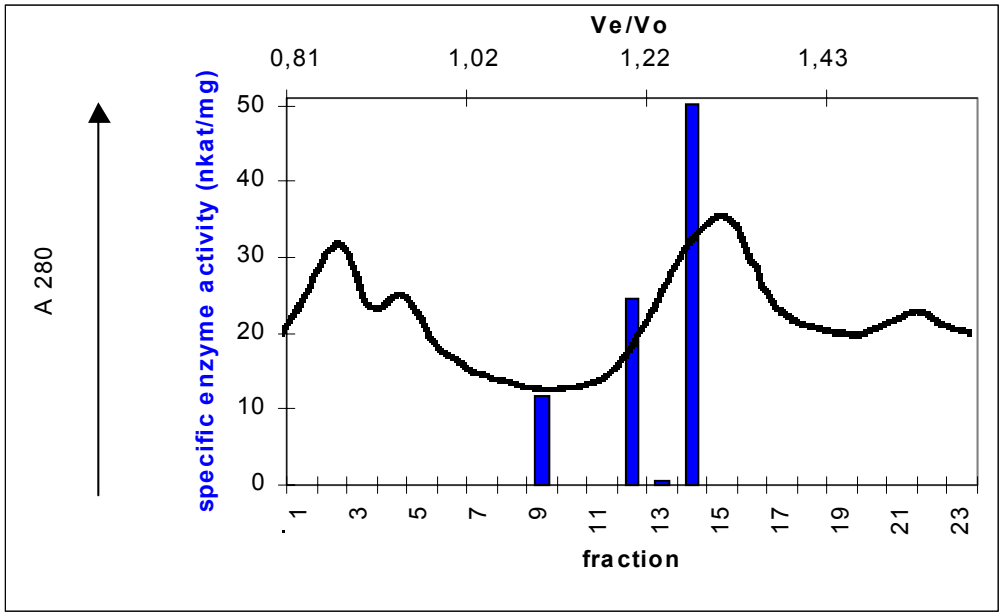


Graph 1: Separation of the cell bound xylanases on CIM® DEAE-8 ml monolithic column. Buffer A: 20 mM TRIS-Cl pH = 7.2; buffer B: buffer A + 1 M NaCl; gradient 0-100% B in 17 minutes; flow rate 6 ml/min. Detection UV-VIS at 280 nm (specific enzyme activity ■).



Graph 2: Gel filtration chromatogram of the sample 4+5 from the anion exchange elution. Elution buffer : 0.1 M NaCl in 20 mM TRIS-Cl pH = 7.2; flow rate 1 ml/min. Detection UV-VIS at 280 nm.

According to the separation chromatogram of the sample 12+13 only some fractions were tested. The highest activity was determined in the fractions 9 and 12. (Graph 3) We expected that applying the fractions 9 to 12 to xylanogram would result in clearing zones. Unfortunately it was not possible to detect any xylanolytically active enzyme from those fractions. This can be due to very low total protein content in the fractions that resulted only in spectrophotometrically detectable activity (according to the Lever method) (Figure 2, lanes 9' to 12').



Graph 3: Gel filtration chromatogram of the sample 12+13 from the anion exchange elution. Elution buffer: 0.1 M NaCl in 20 mM TRIS-Cl pH = 7.2; flow rate 1 ml/min. Detection UV-VIS at 280 nm.

51 and 58 kDa endoxylanases from bulk of proteins in the cell extract were successfully separated. The most active enzyme was the one from the fraction 9 of the sample 4+5. This fraction was also the basis for the yield and purification factor calculation (Table 1).

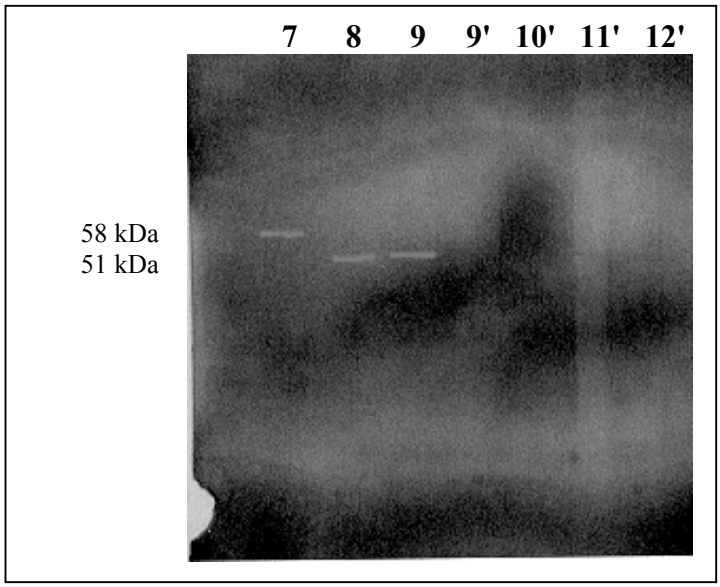


Figure 2: Xylanogram of the chosen fractions from gel filtration of samples 4+5 and 12+13. (see text for the details)

Table 1: Yields and purification factors for the 51 kDa endoxylanase

Fraction	protein concentration *(mg/ml)*	Total protein *(mg)*	Specific activity *(nkat/mg)*	yield *(%)*	purification factor
cell extract	0,214	14,98	21,63	100,00	1,0
AIEX	0,037	2,442	21,02	15,84	1,0
Ultrafiltration	0,079	0,237	34,90	2,55	1,6
GF	0,013	0,065	57,75	1,16	2,7

Comments: AIEX – pooled and concentrated fractions 4 and 5 from the anion exchange chromatography; GF – fraction 9 from the size exclusion chromatography of the sample 4+5.

Conclusions

We assume that we have found a simple and successful procedure for the isolation of xylanases from *Butyrivibrio* sp. strain Mz5. Especially with the respect to the fact that there are not many native bacterial xylanases isolated yet and that in general they are not easy to be isolated.[14] The key isolation point was the use of CIM® DEAE-8 ml monolithic column which allowed the application of non-concentrated cell extract what prevented the otherwise frequent aggregation of xylanases. Relatively low yield and

purification factors could be increased by improving separation conditions. Further experiments for determination of the temperature and pH optima, stability, influence of different ions and chemicals on the enzyme activity and determination of specificity of the purified enzymes are planned.

Acknowledgements

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References and Notes

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Povzetek

Ksilanolitični encimi so pomembni v industriji celuloze in papirja ter prehranski industriji, uporabljamo pa jih tudi kot dodatek krmi ali hrani. Veliko truda je bilo vloženega v iskanje mikroorganizmov, ki so sposobni razgradnje ksilana. Ena izmed najbolj ksilanolitično aktivnih vampnih bakterij je *Butyrivibrio* sp. sev Mz5, ki sintetizira več različnih ksilanaz. V predstavljenem delu je opisan postopek izolacije dveh izmed njih z anionsko izmenjevalno kromatografijo in nadaljnjo gelsko filtracijo. Ključni izolacijski postopek je bila uporaba CIM® DEAE monolitnega modula, s čimer smo se izognili drugače pogosti agregaciji proteinov in tudi pospešili celotni postopek. Izolirana encima imata molekulsko težo 51 in 58 kDa.