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CHITINOLYTIC ENZYMES IN THE RUMEN MICROBIAL ECOSYSTEM

J. KOPEČNÝ[a)] and Blanka HODROVÁ[a)]

[a)] Czech Academy of Sciences, Institute of Animal Physiology and Genetics, Prague 10, Uhříněves, 104 00, Czech Republic, Ph.D., M.Sc., B.Sc.Chem.

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A B S T R A C T

Chitinolytic systems of rumen *Clostridia* (usually the most active rumen chitinolytic bacteria) and rumen anaerobic fungi were compared in the present study. The chitinolytic enzymes of tested *Clostridia* strains were represented mainly by extracellular exochitinases, N-acetylglucosaminidases, chitin deacetylases and chitosanases. Zymograms of exochitinases revealed that these enzymes were bound in complexes with MW higher than 100 kDa. Final products of chitin degradation were glucosamine, N-acetylglucosamine and chitin oligosaccharides. Fungal chitinases were represented mainly by extracellular endochitinases and chitin deacetylase in both mono- and polycentric fungi. The main activities in cellular fraction were chitosanase and endochitinase. There were no or low activity of N-acetylglucosaminidase activity in all fungal strains tested. In comparison with rumen chitinolytic bacteria there was lower production of exochitinases and no production of N-acetylglucosaminidase in rumen fungi.

Key words: microbiology / bacteria / *Clostridium* / fungi / enzymes / chitinolysis / rumen

HITINOLITIČNI ENCIMI V VAMPNEM MIKROBNEM EKOSISTEMU

I Z V L E Č E K

Primerjali smo hitinolitične encimske sisteme vampnih predstavnikov rodu *Clostridium* (običajno najaktivnejše vampne hitinolitične bakterije) in anaerobnih vampnih gliv. Hitinolitične encime proučevanih klostridijev so večinsko predstavljale ekstracelularne eksohitinaze, N-acetilglukozaminidaze, hitinske deacetilaze in hitozanaze. Z eksohitinaznimi encimogrami smo ugotovili, da se ti encimi vežejo v komplekse z MW, večjimi od 100 kDa. Končni produkti razgradnje so bili glukozamin, N-acetilglukozamin in hitinski oligosaharidi. Tako pri mono kot pri policentričnih glivah pa predstavljajo glavne hitinaze predvsem ekstracelularne endohitinaze in hitinske deacetilaze. V celični frakciji je bila največja aktivnost posledica delovanja hitozanaz in endohitinaz. Pri vseh preiskanih sevih smo odkrili le nizko aktivnost (ali pa je spoh nismo zasledili) N-acetilglukozaminidaz. V primerjavi s klostridiji smo pri vampnih glivah odkrili nižjo raven nastajanja eksohitinaz in nič N-acetilglukozaminidaz.

Ključne besede: mikrobiologija / bakterije / *Clostridium* / glive / encimi / hitinoliza / vamp

INTRODUCTION

Chitin is a straight chain homopolymer composed of β-1,4-linked N-acetylglucosamine units with a three-dimensional α-helical configuration stabilized by intramolecular hydrogen bonding which form microfibrils. The most common polymorph is α-chitin, where the polysaccharide chains are arranged in an antiparallel pattern. Chitin microfibrils are covalently or noncovalently associated with various proteins (amoebae, arthropods and nematodes) or polysaccharides (fungi). In crustaceans, calcium carbonate serves as the cementing substance and in some crustacean meals the calcium content reaches 18% of DW. The polymer is analogous to cellulose except that the hydroxyl on the C-2 position of cellulose is replaced by an N-acetylamino group on the chitin molecule. Similar to cellulose chitin forms rigid structures and protects organisms against mechanical damage.

Estimated annual production of chitin is 10^{10}-10^{11}tons in comparison to annual cellulose production - 10^{11}tons. With such large quantities of chitin being produced annually, recycling is important to prevent a sink in the global carbon and nitrogen. Mineralization of chitin is certainly primarily a microbial process. Chitinolytic complex is usually composed of several enzyme activities (Figure 1).

Physiological roles of chitinases could be classified into three categories. The main one is the *digestive or lytic role* of chitinases. The main aim of the activity is to release substrate, in this case glucosamine, for further fermentation. The second role is *autolytic role* of chitinases in organisms synthesizing chitin. Rigid structures of chitin are rebuilded with the help of chitinases and enabling for example fungal mycelium to redirect its growth or insects to rebuild its exoskeleton. Beside that, chitinases are involved in biological interactions. The third role of the activity is therefore *attacking or defending role*. The attacking role is obvious in pathogens of chitin synthesizing organisms - fungi, protozoa and bacteria invading arthropods worms or insects. The defending role of chitinases is the most evident and common in plants where chitinases are produced in case of infection or wounding against invasion of pathogen fungi. In carnivorous plants the defending system has been changed into the digestive role. But the defending role of chitinases was observed in animals as well. Chitinolytic activity was observed in the blood serum of ruminants and turbot.

But the general model for defending antifungal complex is considered the plant one. There is not only important activity of endochitinase but also presence of lysozyme and β-1,3-glucanase activity. Only this combination is able to stop fungal spore germination and reduce the growth of mycelium.

Chitinolytic enzymes are constituents of all microbial types in the rumen microbial ecosystem. The digestive role of these enzymes was recognized in bacteria (Morgavi *et al*., 1994) and protozoa (Widyastuti *et al*., 1995). On the opposite, fungal chitinases play mainly a morphogenetic role. Their presence is essential for normal hyphal growth and branching (Gooday, 1990).

Protozoa

Morgavi *et al*. in 1993 showed that rumen protozoa possessed complex chitinolytic activity and their enzymes could hydrolyze anaerobic fungi - *Neocallimastix* and *Piromyces* strains with the production of glucosamine and free amino acids. The same authors later described the enzymatic complex. Rumen protozoa produced mainly *exochitinase* and *N-acetyl-β-glucosaminidase*. Activities of lysozyme and 1,3-β-glucanase which could be involved in fungal chitin degradation were also present. Intracellular chitinolytic activities in protozoa were about three times higher than the activity of bacterial origin. On the other hand the protozoal activity was available only to engulfed substrate that means mainly to fungal spores.

Fungi

As anaerobic fungi produce their cell walls basically with chitin, they need not only an enzymatic system for chitin synthesis but also a system for chitin modification. Sakurada *et al*. (1995) found traces of exochitinase (substrate PNP-diacetylchitibiose) and dominant endochitinase activity in *Piromyces communis* OTS1. The aim of this study was to describe the differences in chitinolytic systems in rumen bacteria and fungi.

MATERIAL AND METHODS

Chemicals

Colloidal chitin was prepared from crab shell chitin (Sigma, USA) according to the method of Shimahara and Takiguchi (1988). Carboxymethyl-chitin was prepared according to the method of Wirth and Wolf (1990) from the same practical grade chitin.

Micro-organisms

Clostridium tertium strain ChK5 was isolated from the rumen fluid of cow (Kopečný *et al*., 1996a). Strains *Clostridium sp.*,12, 13 and 14 were isolated from the rumen fluid of sheep fed with meadow hay and barley. Isolation was done under anaerobic conditions. Rumen fluid samples were incubated with liquid medium M10 with 0.4% (w/v) colloidal chitin, enriched chitinolytic cultures diluted and grown on the same medium with 1.5% (w/v) agar on petri dishes. Clearing zones of hydrolyzed chitin appeared after 3-5 days. Isolated strains were cultivated in medium M10 with colloidal chitin or glucose and further characterized. Isolation of chitinolytic bacteria and anaerobic fungi from faeces of ZOO animals was done in the same manner. Fresh samples were transferred at 35-40^{o}C into anaerobic liquid medium M10 and transported in 2 hours time to our lab where they were reinoculated. Chitinolytic bacteria were enriched on medium M10 with colloidal chitin (4mg/ml) and anaerobic fungi on medium M10 with 30% of rumen fluid, 4 mg/ml of cellobiose and a mixture of antibiotics (Kopečný, 1995).

Cultivation methods

Chitinolytic strains of rumen bacteria were cultivated in modified medium M10 with 0.5% (w/v) colloidal chitin (Kopečný *et al*., 1996a). Anaerobic fungi were grown in the same medium with (4 mg/ml) microcrystalline cellulose or cellobiose (Kopečný *et al*., 1996b). Both, bacterial and fungal cells were spun down (8500xg, 10 minutes, 4^{o}C) and resuspended in distilled water (1/10 of the original volume). Supernatant was concentrated 10 x by ultrafiltration (Amicon, PM.10, MW cutoff 10 kDa).

Analytical methods and enzyme assays

Total chitinase (joint exo- and endo-) activity was determined with 0.5% (w/v) colloidal chitin as a substrate and reducing groups were detected with p-hydroxybenzoic acid hydrazide (Lever, 1977). Exochitinase was measured with PNP-β-D-N,N`-diacetylchitobiose according to Roberts and Selitrennikoff (1988). Chitin deacetylase was measured with colloidal chitin and produced chitosan estimated with 3-methyl-2-benzothiazoline hydrazone (Kauss and Bauch, 1988). Chitosanase activity was estimated with colloidal chitosan and reducing sugars estimated with p-hydroxybenzoic acid hydrazide (Lever, 1977). N-acetyl-β-glucosaminidase was assayed with p-nitrophenyl-β-N-acetyl-glucosaminide (pNAG, Sigma) according to Bidochka at al., (1992). Lysozyme activity was estimated spectrophotometrically by measuring the decrease in optical

density at 660 nm (Wang and Chang, 1997). Endoglucanase activity was measured with 0.5% (w/v) carboxymethyl cellulose as a substrate, and released reducing sugars were detected by the method of Lever (1977). All enzyme activities were measured at least in triplicates if not stated otherwise. These activities were expressed in levels present in culture medium.

High performance liquid chromatography (HPLC)

The HPLC system consisted of a P2000 pump, an AS3500 sampler, a SpectraFocus scanning detector (SpectraPhysics, Fremont, CA) and a Shodex RI-71 detector (Showa Denko, Japan). Obtained results were evaluated with a PC1000 SpectraSystem software. Sugars and organic acids were separated on an ion exclusion Ostion LG KS 0800 H^+ column (4 x 250 mm, 65^oC, Tessek, Prague, Czech Republic) using 5 mM H_2SO_4 as the mobile phase, at a flow rate 0.4 ml per min. Saccharides were monitored by refractive index and also by the detection of the absorbance at 190 nm.

RESULTS AND DISCUSSION

Bacteria

We have tried to correlate the presence of anaerobic fungi with the presence of chitinolytic bacteria in the digesta of herbivorous animals. At the beginning of experiments - in case of domestic ruminants - the correlation was evident. But in the case of ZOO animal faeces the correlation was lost. The presence of chitinolytic bacteria was more widespread than the presence of fungi as can be seen from Table 1.

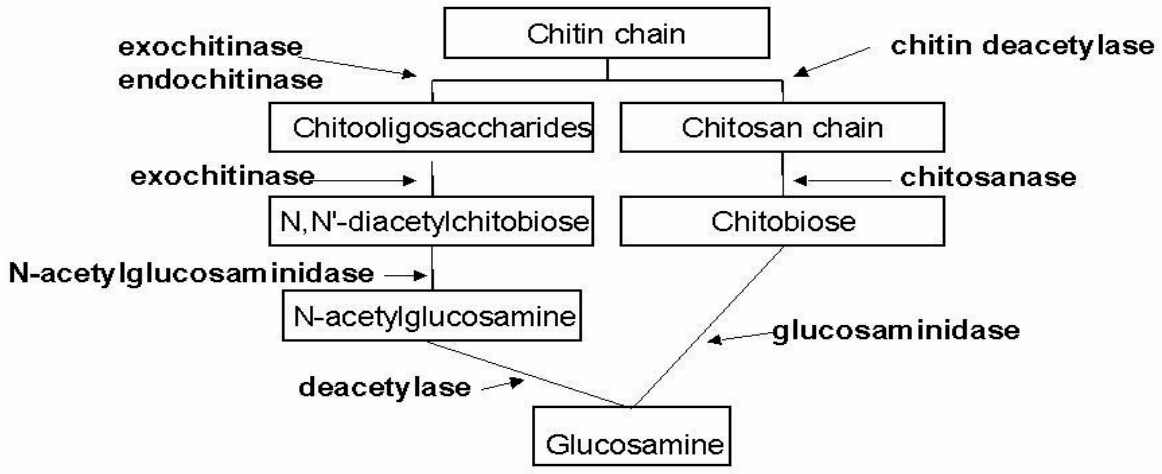

Figure 1. Pathways of chitin degradation

In ruminants we found that chitinolytic activity was produced mainly by clostridia-like strains (Kopečný *et al*., 1996a). Therefore we chose four strains from the rumen fluid of sheep and cow to demonstrate the composition and localization of the enzymes involved in the chitinolytic complex. The strain CHK5 was classified as a *Clostridiun tertium.* Other strains were not further characterized. In the first step the activity was measured against colloidal chitin (Table 2). The activity was mainly extracellular. This activity was shown to be exo-type, which is typical for bacteria. This enzyme splits off disaccharides from the non-reducing end of the polysaccharide chain.

Table 1. Presence of chitinolytic bacteria and anaerobic fungi in digesta of herbivorous animals.

Animal	Presence of	
	anaerobic fungi[a]	chitinolytic bacteria[b]
Cattle	+	+
Sheep	+	+
Antelope nigalo	-	+
Antelope bongo	+	+
Bison	+	+
Red kangaroo	-	+
European elk	-	+
Llama guanaco	+	+
Takin	+	+
Sika deer	-	+
Zebra Grevy	+	+
Bactrian camel	-	+
European bison	+	+
Rotschild giraffe	+	+

[a] - Anaerobic fungi were enriched on medium M10 in the presence of antibiotics.
[b] - Chitinolytic bacteria were enriched on the same medium but the substrate was 0.5% colloidal chitin.

Table 2. Activity of chitinases in the extracellular and intracellular fractions of different rumen chitinolytic bacteria.

Strain	Chitinase activity[a] (μg N-acetylglucosamine/ml/h)		K_m[b] (mg/ml)
	Cells	Medium	
ChK5	4.22 ± 2.13	46.17 ± 0.63	0.055
OV12	0	34.38 ± 1.66	0.079
OV13	3.12 ± 1.61	41.87 ± 1.48	0.038
OV14	16.48 ± 2.16	14.54 ± 1.43	0.134

[a] - Colloidal chitin was used as a substrate
[b] - Michaelis constant was estimated with extracellular enzymes.

Usually the hydrolysis product- diacetylchitobiose - is transported into the cell and fermented. In our isolates the activity of N-acetylglucosaminidase was surprisingly also exocellular (Table 3). In ovine isolates the activity was lower but also mainly present in the culture medium.

The chitinolytic complex of above mentioned bacteria contained also chitosanase activity (Table 4). This activity was of course exocellular and was several times higher in ovine clostridia. This phenomenon correlated with higher deacetylase activity in ovine isolates (Table 5). It was probable that these selected isolates preferred chitosan branch in the chitin degradation especially in the ovine isolates (Table 1). Chitinases in these bacteria - OV 12, 13 and 14 showed different molecular masses in native PAGE (Table 6).

Table 3. Activity of N-acetylglucosaminidase in the extracellular and intracellular fractions of different rumen chitinolytic bacteria.

	N-acetylglucosaminidase activity[a] (μg N-acetylglucosamine/ml/h)	
Strain	Cells	Medium
ChK5	3.40 ± 0.45	48.00 ± 4.42
OV12	0.03 ± 0.10	3.70 ± 0.47
OV13	1.90 ± 0.12	5.80 ± 0.29
OV14	1.03 ± 0.59	2.60 ± 0.27

[a] - PNP N-acetyl-β-D-glucosaminide was used as a substrate.

Table 4. Activity of chitosanase in extra and intracellular fractions of different rumen chitinolytic bacteria.

	Chitosanase activity[a] (ng glucosamine/ml/h)		Endoglucanase activity[b] (ng glucose/ml/h)
Strain	Cells	Medium	Medium
ChK5	38.3 ± 3.5	482.2 ± 55.8	420 ± 10
OV12	153.3 ± 24.6	921.6 ± 77.0	0
OV13	1036.8 ± 98.5	2841.6 ± 93.7	0
OV14	1190.3 ± 109.7	5145.6 ± 187.9	2000 ± 200

[a] - Colloidal chitosan was used as a substrate. [b] - Carboxymethyl cellulose was used as a substrate.

Table 5. Activity of chitin deacetylase in extra and intracellular fractions of different rumen chitinolytic bacteria.

	Chitin deacetylase activity[a] (ng glucosamine/ml/h)	
Strain	Cells	Medium
ChK5	114 ± 31	381 ± 63
OV12	350 ± 89	279 ± 44
OV13	88 ± 14	2009 ± 112
OV14	205 ± 45	455 ± 33

[a] - Colloidal chitin was used as a substrate and released amino groups measured.

The chitinolytic complex of Cl. tertium CHK5 was further characterized. Optimal pH for the most enzymes was close to 7 with the exception of exochitinase with the highest activity in the range of 4.5 to 6.1 (Table 7). We did not find any specific inhibitor (Table 8) but exochitinase was stimulated by reducing compounds. This feature is not common in bacterial chitinases and the only SH- sensitive system in anaerobic bacteria, connected with chitin degradation was described for the transport of diacetyl chitobiose into Clostridium 9.1 strain (Pel and Gottshal, 1987).

Table 6. Preliminary estimated assignation of chitinolytic bacteria and molecular mass of their chitinolytic complex.

Isolate (kDa)[c]	Source	Assignation	MW of chitinase complex
ChK5	cow[a]	*Clostridium tertium*	420 000
OV12	sheep[a]	*Clostridium sp.*	550 000
OV13	sheep[a]	*Clostridium sp.*	550 000
OV14	sheep[a]	*Clostridium sp.*	550 000
Wel 2	camel[b]	*Clostridium sp*.	ND
AV 2	antelope[b]	*Clostridium sp*.	ND

[a] - isolated from the rumen fluid. [b] - isolated from faeces.
[c] - molecular mass was estimated with native electrophoresis on PAG and the zymogramm

Table 7. Optimal pH for different chitinolytic enzymes of *Cl. tertium* ChK5.

Strain	Enzyme	pH optima [a]
ChK5	exochitinase	4.5 - 6.1
	Chitosanase	6.3
	N-acetylglukosaminidase	7.4
	Chitin deacetylase	7.0

[a] - pH optima were established with 100mM citrate-phosphate buffer in the interval from pH 3 to pH 8.

Table 8. The effect of inhibitors on the activity of exocellular chitinase from *Cl. tertium* ChK5

Inhibitor	Concentration (mM)	Activity of chitinase[a] (%)
Control	-	100.0
Cysteine	5	205.0
EDTA	5	107.8
Fenantroline	5	115.5
SDS	5	36.0

[a] - chitinase activity was measured in 10 mM phosphate buffer pH 7,0 with colloidal chitin at 39°C. Activity of the control was 327 g N-acetylglucosamine/h/ml.

The presence of β-1,3-glucanase and lysozyme activity was necessary for a classical antifungal system as found earlier (Ji and Kuc, 1996, Terwisscha *et al*., 1994). The strain CHK5 was proved to posses the lysozyme activity but the β-1,3-glucanase was present in traces only (Table 9).

Fungi

Two strains of anaerobic fungi – polycentric *Orpinomyces joyonii* A4 and monocentric *Neocallimastix patriciarum* Cx (Table 10) were tested. Both showed most of chitosanase and endochitinase activity in cell fractions. Most of the deacetylase activity was found in medium. Polycentric isolate produced significantly more endochitinase while the monocentric one exhibited higher activity of chitosanase. In *Orpinomyces joyonii* A4 culture β-1,3-glucanase but

no exochitinase activity was proved (Table 11). The only common feature of fungal cultures was no production of N-acetylglucosanimidase activity.

Table 9. Lysozyme, exochitinase and β-1,3-glucanase activity in the culture of *Cl. tertium* CHK5

Activity	Activity unit	Fraction	
		Exocellular	Endocellular
lysozyme[1]	U/min./ml		
CHK5		33.2	ND
β-1,3-glucanase[2]	μg glucose/h/ml		
CHK5		1.8	0
OV 12		5.0	ND
OV 13		2.9	ND
OV 14		1.2	ND
exochitinase[3]	μg N-ac.glukoseNH_2/h/ml		
CHK5		70.2	25.7
OV 12		77.3	ND
OV 13		77.6	ND
OV 14		76.7	ND

[1]- substrate - suspension of *Micrococcus lysodeikticus* cells (5mg /ml).
[2]- substrate - laminarin (5 mg /ml).
[3]- substrate - PNP-β-D-N,N`- diacetylchitobiose (5mg/ml).
ND - not determined

Table 10. Activity of chitinolytic enzymes in anaerobic fungi.

Anaerobic fungus	Localization	Deacetylase	Chitosanase	Endochitinase
		(ng N-acetylglucosamine/h/ml)		
Orpinomyces joyonii A4	cell wall	2488	4860	12270
	cell extract	49	12540	26373
	medium	5769	0	27600
Neocallimastix patriciarum (	cell wall	0	12290	0
	cell extract	0	410	3680
	medium	269	845	1226

The differences in chitinolytic complexes of rumen bacteria and fungi will be used for the description of the negative influence of these bacteria on rumen fungi.

Table 11. Exochitinase and β-1,3-glucanase activity in the culture of *Orpinomyces joyonii* A4

Activity	Activity unit	Exocellular activity
β-1,3-glucanase[1]	μg glucose/h/ml	769
Exochitinase[2]	μg N-ac.glucoseNH_2/h/ml	0

[1]- substrate - laminarin (5 mg /ml).
[2]- substrate - PNP-β-D-N,N`- diacetylchitobiose (5mg/ml).

SUMMARY

1. The presence of chitinolytic bacteria was proved in digesta of most herbivorous animals tested.
2. Chitinolytic strains of bacteria were isolated from the rumen fluid of sheep and cow.
3. Chitinolytic complex of these isolates was characterized.
4. Properties of above mentioned enzymes were compared with chitinolytic complex produced by anaerobic fungi.

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