ELSEVIER

Journal of Chromatography B, 758 (2001) 197–205

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components

Pirkko Helistö[a], Gleb Aktuganov[b], Nailia Galimzianova[b], Alexander Melentjev[b], Timo Korpela[a,*]

[a] *Joint Biotechnology Laboratory, University of Turku, BioCity 6A, 20520 Turku, Finland*
[b] *Institute of Biology of the Ufa Research Center, Russian Academy of Sciences, Ufa, Bashkorstan 450054, Russia*

Received 17 October 2000; received in revised form 5 March 2001; accepted 16 March 2001

Abstract

Bacillus sp. X-b, a biocontrol agent against certain plant pathogenic fungi, secretes a complex of hydrolytic enzymes, composed of chitinase, chitosanase, laminarinase, lipase and protease. Homogenized mycelium of basidiomycete *Macrolepiota procera* induced activities of these enzymes more effectively than colloidal chitin or partially purified cell walls of another basidiomycete *Polyporus squamosus*. Subjected to a multi-step purification, the specific activity of chitinase increased 36-fold, chitosanase 69-fold, lipase 44-fold and laminarinase 15-fold. Partially purified chitinase showed two major bands with molecular masses of 46 000 and 35 000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis while chitosanase and lipase appeared as single bands with molecular masses of 27 000 and 62 000, respectively.

Keywords: *Bacillus* sp. X-b; Lytic enzymes

1. Introduction

Microorganisms, which secrete a complex of mycolytic enzymes, are considered to be possible biological control agents of plant diseases or as bioprotectants of wood decay [1–7]. Lorito et al. deduced that chitinolytic enzyme complex from *Trichoderma harzianium* plays the major role in mycoparasitism of the fungus and may be used as an alternative or second mode of biological control [8]. Bacteria, belonging to the genus *Bacillus* are known as antagonists of many mycelial fungi [9–11]. It is known also that *Bacillus* spp. strains are able to secrete a number of hydrolytic enzymes, including chitinases, laminarinases, and cellulases [1,12–14]. However, the antagonistic role of complexes of bacillar hydrolases against fungi is not yet understood. It is often supposed that chitinase and laminarinase are important for the antagonism but possibly also other hydrolases are important for the source of lysis of fungal cell walls [12].

We describe here isolation and partial purification of hydrolases from cultivation medium of *Bacillus* sp. X-b, an antagonist of certain wood destroying

*Corresponding author. Tel.: +358-2-3338-066; fax: +358-2-3338-080.
E-mail address: timokor@utu.fi (T. Korpela).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00181-5

mycelial fungi. The results imply that the enzymes are at least partially involved in the antagonism.

2. Materials and methods

2.1. Microbial cultures

Bacillus sp. X-b from the Collection of Microorganisms at the Institute of Biology, Ufa Scientific Center, Russian Academy of Sciences, was originally isolated from soil as a producer of chitinase (chitinase and chitosanase activities 49 U and 94 U, respectively). This strain also showed inhibition of growth of certain fungi that might initiate colonization process of wood decay. The culture was maintained on nutrient agar, containing (w/v) 1.0% colloidal chitin (from crab shells), 0.3% polypeptone, 0.1% corn steep liquor, 0.1% of KH_2PO_4, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2 \cdot 2H_2O$, 1.6% agar, at pH 6.5–6.7. The microbe was cultivated at 37°C for 72 h on a rotary shaker (165 rpm) in the flasks with liquid medium (pH 6.5–6.7) containing 0.3% polypeptone, 0.1% corn steep liquor, 0.1% KH_2PO_4, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2 \cdot 2H_2O$. Colloidal chitin (1%) or 0.5% disintegrated fruit bodies of *Macrolepiota procera*, or 0.5% partially purified cell walls of *Polyporus squamosus* was added as chitin-containing substrates. Chitin and microbial growth media were purchased from Sigma, USA. The salts were of analytical grade.

2.2. Preparation of colloidal chitin and colloidal chitosan

Colloidal chitin was prepared by a modification of the method of Rodriguez-Kabana et al. [15]. For preparation of colloidal chitosan, 5 g of chitosan (Sigma, with 18% *N*-acetylation) was dissolved in 100 ml of 1 *M* HCl with continuous stirring while heating from room temperature to 50–60°C during 30–40 min. The pH of solution was maintained at 6.5–7.0 by addition of 50% (w/v) NaOH. The precipitate was washed 8–10 times with distilled water, collected by centrifugation and used in the later experiments.

2.3. Enzyme assays

The activities of chitinase, chitosanase and laminarinase were determined by measuring of the release of reducing sugars by a modification of the Schales method [16]. One enzyme unit of these enzymes was defined as 1 μmol of *N*-acetyl-D-glucosamine, D-glucosamine, or D-glucose formed, respectively, per ml of the reaction mixture during 1 min at 55°C. The reaction mixture for chitinase and chitosanase contained 0.6 ml of enzyme solution in sodium phosphate buffer (25 m*M*, pH 6.0) and 0.3 ml of 1% (w/v) suspension of colloidal chitin or 5% (w/v) suspension of colloidal chitosan in the same buffer. The reaction was carried out at 55°C for 1 h. The reaction mixture for laminarinase contained 0.25 ml of enzyme solution in sodium phosphate buffer (25 m*M*, pH 6.0) and 0.25 ml of 0.2% soluble laminarin (Sigma) in the same buffer.

Lipase activity was determined by the release of *p*-nitrophenol from *p*-nitrophenylbutyrate (*p*-Np-butyrate) [17]. One unit of lipase activity was defined as 1 μmol of *p*-nitrophenol formed per ml of reaction mixture during 1 min at 37°C. The reaction mixture for lipase contained 0.89 ml of Tris–HCl buffer (0.5 *M*, pH 7.0), 0.10 ml of enzyme solution and 10 μl of 100 m*M* *p*-Np-butyrate (Sigma). The reaction mixture was pre-incubated without substrate at 37°C for 10 min. The substrate solution was added and kinetics at 405 nm was recorded for 3 min.

Protease activity was assayed with the method by Haab et al. [18].

Proteins were determined according to Bradford [19] with bovine serum albumin as standard.

2.4. Preparation of crude enzyme

Culture liquid was centrifuged at 10 500 *g* for 15 min for removal of cells and the remainder of the substrates. Supernatant was saturated with solid ammonium sulfate to 65% (w/v) and allowed to precipitate in an ice-bath. The solids were collected by centrifugation at 10 500 *g* (30 min), redissolved in 50 ml of 0.025 *M* sodium phosphate (pH 6.0) and dialysed against 3 l of the same buffer overnight.

Ion-exchange chromatography was performed on a column (100×20 mm) of DEAE-Sepharose (Pharmacia) equilibrated with Tris–HCl (25 m*M*, pH 7.1).

A linear gradient of 0–1.0 *M* NaCl in the same buffer was applied to elute proteins (flow-rate 40 ml/min). Fractions of 10 ml were collected.

A column (530×27 mm) of Bio-Gel P-100 (Bio-Rad) was equilibrated with sodium phosphate (25 m*M*, pH 6.0) with a flow-rate of 0.2 ml/min. The proteins were eluted within the same conditions and fractions (5 ml) were collected.

''Microsep FILTRON'' centrifugal concentrators with a molecular mass (M_r) cutoff of 10 000 were employed for the concentration of enzymes.

2.5. Gel electrophoresis

Standard sodium dodecyl sulfate (SDS) gel electrophoresis techniques were applied with a 4% stacking gel and a 12.5% separating gel on a mini vertical slab gel system ''Mini-Protean II Electrophoresis Cell'' (Bio-Rad). The SDS–polyacrylamide gel electrophoresis (PAGE) protein molecular mass standards (Pharmacia) were ovotransferrin (76 000–78 000), bovine serum albumin (66 250), ovalbumin (43 000), carbonic anhydrase (bovine, 30 000), equine myoglobin (17 200) and cytochrome *c* (12 300).

3. Results

3.1. Influence of chitinous substrates on the spectrum and activity of hydrolytic enzymes of Bacillus sp. X-b

It was demonstrated previously [10,20,21] that material from fruit bodies of *Basidiomycetes* induces secretion of mycolytic enzymes on bacilli. Therefore disintegrated fruit bodies of *M. procera* and *P. squamosus* at different concentrations were added into the growth medium to induce activities of the enzymes. Production of extracellular enzymes was determined after a 72-h induction in liquid medium (Table 1). The widest spectrum of hydrolytic enzymes and highest enzyme activities was found in the medium containing material from *M. procera*. The protease activity was increased 15-fold and laminarinase activity twofold. This culture liquid was employed in the purification studies.

3.2. Purification of chitinase

DEAE-chromatography of crude chitinase resulted in chitinase which also contained laminarinase and protease (Fig. 1). In order to avoid these accompanying activities an affinity adsorption method was applied: 4.5 g of moist colloidal chitin was added to 45 ml of crude enzyme preparation. The suspension was agitated in an ice bath with a magnetic stirrer for 90 min. Chitin with adsorbed proteins were collected by centrifugation (30 min at 12 000 *g*). The pellet was washed twice with 40 ml of cold sodium phosphate (25 m*M*, pH 6.0) followed by 20 ml of the same buffer added to the suspension. This mixture was incubated at 50°C for 15 h and the non-hydrolyzed chitin removed by centrifugation (15 min at 12 000 *g*). The supernatant was dialyzed against 2 l of Tris–HCl (25 m*M*, pH 7.1) for 12 h. The dialysate was chromatographed on DEAE-Sepharose (Fig. 2). The fractions with chitinase activity were pooled and concentrated. The results of purification are summarized in Table 2.

Distributions of several enzyme activities between soluble and chitin-bound fractions are presented in Table 3. The material adsorbed onto colloidal chitin contained mainly chitinase and only small amounts

Table 1
Effect of main carbon sources on production of lytic enzymes by *Bacillus* sp. X-b

Enzyme (U/ml)	Chitin-containing substrate (%, w/v)		
	Colloidal chitin, 1.0%	*Macrolepiota procera*, 0.5%	Purified cell walls from *Polyporus squamosus*, 0.5%
Chitinase	0.04	0.07	0.05
Chitosanase	0.00	0.25	0.03
Laminarinase	1.55	2.40	1.35
Lipase	nd	6.00	nd
Protease	0.08	1.20	0.08

nd, Not determined.

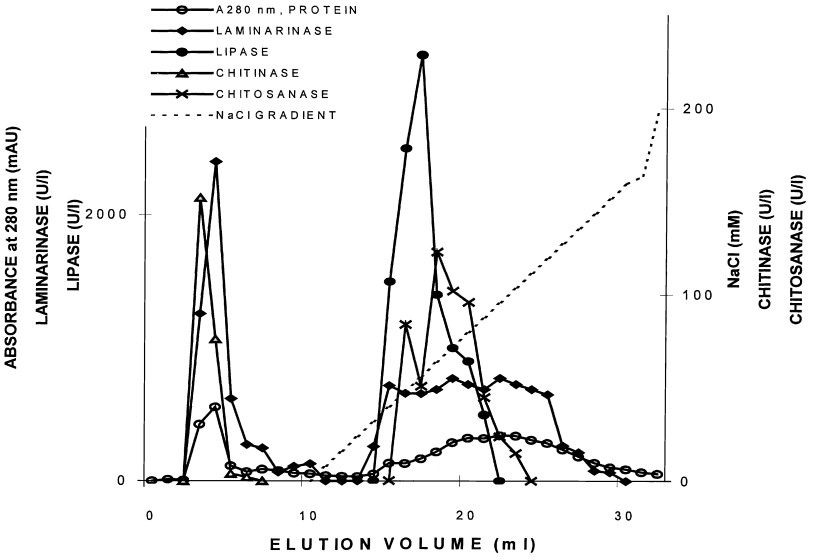

Fig. 1. Ion-exchange chromatography of a crude chitinase from *Bacillus* sp. X-b on DEAE-Sepharose. Mobile phase was 50 m*M* Tris–HCl, pH 7.1.

of other enzymes. DEAE-chromatography separated chitinase from the other studied enzyme activities. Nevertheless, SDS–PAGE of the enzyme showed that chitinase was not, however, homogeneous but contained two major protein bands with M_r 46 000 and 35 000 (Fig. 3).

3.3. Purification of other hydrolytic enzymes

Chitosanase activity did not bind onto colloidal chitin (Table 3) and its further purification was performed from this fraction (Table 4). Affinity adsorption onto colloidal chitosan enabled us to

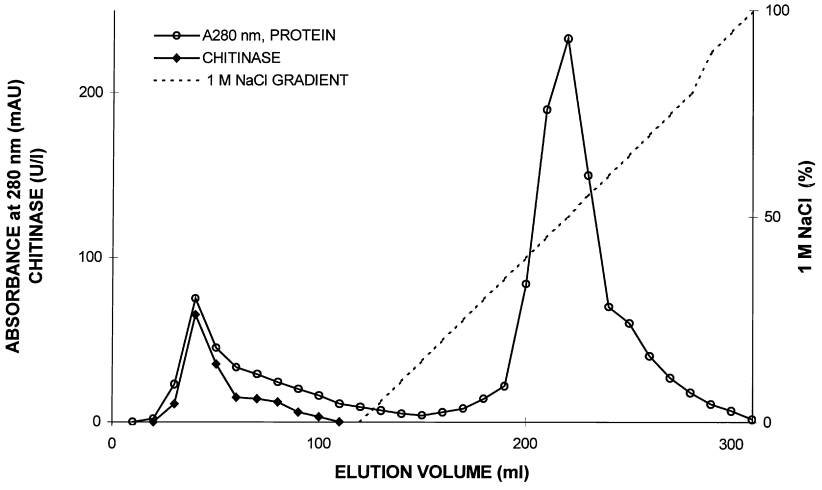

Fig. 2. Anion-exchange chromatography of chitinase from *Bacillus* sp. X-b on DEAE-Sepharose. The enzyme was previously adsorbed on colloidal chitin. Mobile phase was 50 m*M* Tris–HCl, pH 7.1.

Table 2
Purification of chitinase from *Bacillus* sp. X-b

Step	Total chitinase (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
1. Culture supernatant	49	1323	0.04	1	100
2. Ammonium sulfate 65%	21	771	0.03	0.8	44
3. Adsorbtion on colloidal chitin	5.1	41	0.12	3.3	9.5
4. DEAE-Sepharose	0.95	0.71	1.35	36	2.0

Table 3
Distribution of lytic enzymes after adsorption on colloidal chitin

Enzyme	Enzyme activity adsorbed on chitin			Enzyme activity not adsorbed on chitin		
	U/ml	U/mg[a]	%[b]	U/ml	U/mg[a]	%[b]
Chitinase	0.22	0.12	9.5	0.08	0.01	9.7
Chitosanase	0.10	0.06	1.4	0.82	0.10	53
Laminarinase	2.7	1.51	3.7	21	2.8	78
Lipase	18	10.1	9.5	18	2.3	25
Protease	0.05	0.03	0.1	4.0	0.51	29

[a] Specific activity.
[b] Recovery from total enzyme activity.

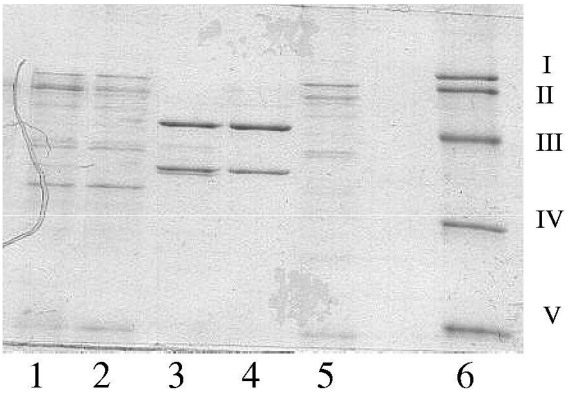

Fig. 3. SDS–PAGE of chitinase preparations from *Bacillus* sp. X-b. Lane 1: concentrated cultural broth after precipitation with ammonium sulfate and dialysis; lane 2: non-adsorbed fraction of the same preparation after subjecting to colloidal chitin; lane 3: chitinase adsorbed on colloidal chitin; lane 4: chitinase adsorbed onto colloidal chitin followed by chromatography on DEAE-Sepharose with showing two major bands at M_r 46 000 and 35 000; lane 5: fraction of proteins non-adsorbed onto colloidal chitin and chitosan; lane 6: molecular mass markers (I–V) ovotransferrin, bovine serum albumin, ovoalbumin, carbo-anhydrase, and equine myoglobin, respectively.

remove bulk impurities and other compounds since chitosanase was not adsorbed onto its substrate. Ion-exchange chromatography separated chitosanase into two fractions: unadsorbed and adsorbed onto DEAE-Sepharose (Fig. 4). The second (adsorbed) fraction of chitosanase was eluted with linear gradient of 0–0.5 *M* of NaCl and located within fractions 48–63 (Fig. 4). Laminarinase and lipase activities were detected also in these fractions. They were concentrated and rechromatographed on DEAE-Sepharose at pH 8.0. In these conditions all enzymes were adsorbed on column and were then eluted by linear gradient 0–0.2 *M* of NaCl. Lipase eluted out as one peak and laminarinase and chitosanase eluted together (Fig. 5). The pooled fractions of lipase showed a single band with M_r 62 000 on SDS–PAGE (Fig. 6).

For separation of chitosanase and laminarinase, fractions pooled after re-chromatography were concentrated and applied on a column of Bio-Gel P-100. Chitosanase eluted as a separate peak in fractions 15–19 (Fig. 7) and was was a single band with M_r 27 000 on SDS–PAGE. Laminarinase activity was not detected after this step. Apparently it was denaturated or more probably adsorbed by the gel

Table 4
Purification of chitosanase (1), laminarinase (2) and lipase (3) from *Bacillus* sp. X-b

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
1. Culture supernatant	94(1) 1680(2) 4200(3)	1323	0.13 1.27 3.17	1	100
2. Ammonium sulfate 65%	64(1) 1477(2) 2196(3)	771	0.08 1.91 2.85	0.6 1.5 0.9	68 88 51
3. Adsorbtion on colloidal chitin[a]	37(1) 970(2) 810(3)	352	0.10 2.76 2.30	0.8 2.2 0.7	39 58 20
4. Adsorbtion on colloidal chitosan[a]	25(1) 520(2) 440(3)	134	0.19 3.38 3.28	1.4 2.7. 1	27 31 10
5. DEAE-Sepharose	7(1) 132(2) 418(3)	40	0.18 3.33 10.6	1.4 2.6 3.3	7.6 7.8 9.6
6. DEAE-rechromatography	0.4(1) 2.2(2) 28(3)	0.41 0.31 0.20	0.88 7.03 140	6.5 5.5 44	0.4 0.3 0.6
7. Bio-Gel P-100	0.2(1)	0.02	9.31	69	0.3

[a] Fraction not adsorbed.

matrix. Purification of laminarinase and lipase are summarized in Table 4.

4. Discussion

The main function of bacterial extracellular hydrolases is, undoubtedly, the release of nutrients from different substrates for the needs of a bacterium. In addition, excreted enzymes alone, or with other compounds like antibiotics, may be used by bacteria for competition with other microbial species. These two functions of extracellular enzymes are not necessarily controversial but form an ecological box for a bacterium and hence maintain a microbial balance. Especially, bacterial chitinases and β-glucanases are considered as biological control agents of various plant pathogenic or wood deteriorating fungi [3–6,10,22].

Bacillus sp. X-b is a typical antagonist of certain mycelial wood deteriorating fungi and is able to produce a complex of hydrolases when grown in a medium of chitin-containing substrates, which induce the appearance of at least chitinase, chitosanase, laminarinase, lipase and protease. These enzymes were studied here because they are thought to be involved in antifungal mechanism of bacterial antagonists [3,4,6,12].

Pleban et al. [22] found chitinase in an endophytic bacterium *Bacillus cereus* 65 that significantly protected cotton seedlings from root rot disease caused by *Rhizoctonia solani*. A crude preparation of these extracellular enzymes decreased 25% of spore germination of *F. oxysporum* f.sp. Inbar and Chet [3] showed that chitinase of *Aeromonas caviae* is involved in biological control of soil-borne plant pathogens. Lim et al. [4] obtained similar results for the antifungal mechanism of *Pseudomonas stutzeri*

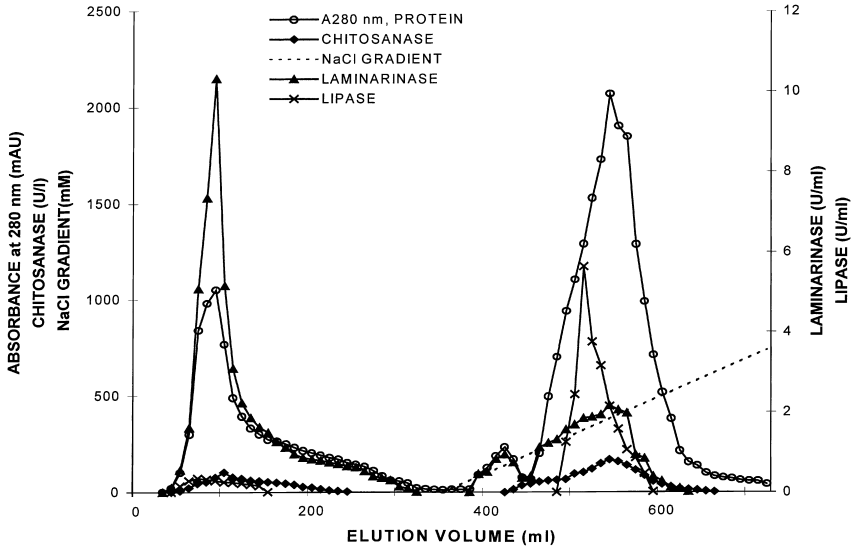

Fig. 4. Anion-exchange chromatography of a crude enzyme preparation on DEAE-Sepharose after adsorption on chitin and chitosan. Mobile phase was 50 m*M* Tris–HCl, pH 7.1.

YPL-1 against *F. solani*. Antifungal activities of the original strain and its chitinase-reinforced or chitinase-defective mutants were then compared. Chitinase-defective mutants of *P. stutzeri* YPL-1 lost the main part of its antifungal activity, while the reinforced mutants showed higher antifungal activities than the original strain. In the course of selection of glucanolytic bacteria from barley rhizospheres, Nielsen and Sørensen [12] showed that fungal antagonism appeared only within those *Bacil-*

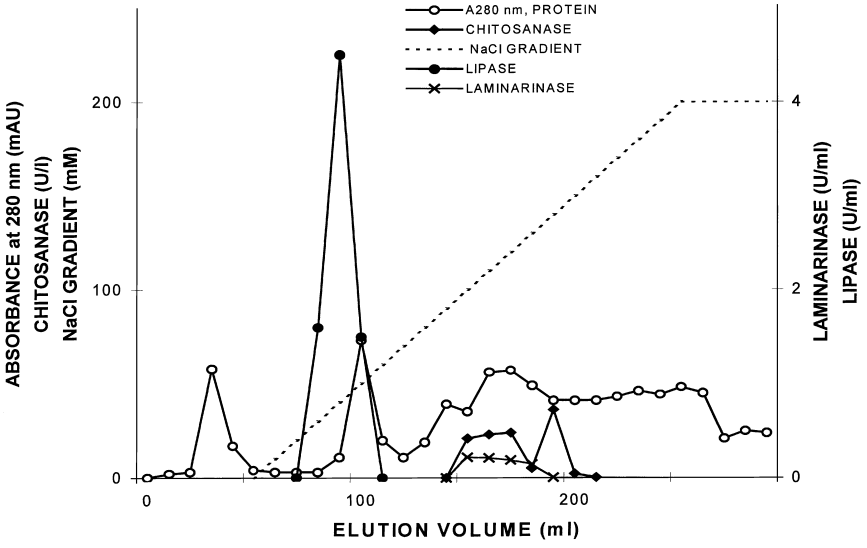

Fig. 5. Rechromatography of fractions containing chitosanase, laminarinase, and lipase on DEAE-Sepharose. For conditions, see Materials and methods.

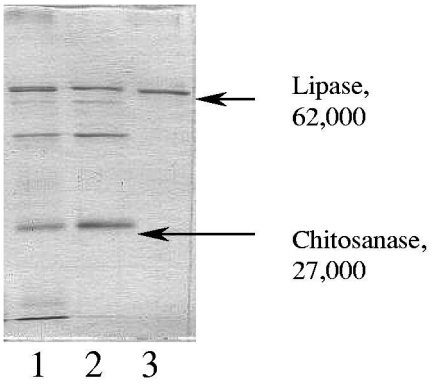

Fig. 6. SDS–PAGE of purified lipase and chitosanase. Lane 1: sample from DEAE-chromatography of enzymes non-adsorbed onto colloidal chitin and chitosan (fraction numbers 48–63 with NaCl gradient 0–1.0 *M*); lane 2: rechromatography on DEAE-Sepharose (Tris–HCl, pH 8.0; NaCl gradient 0–0.2 *M*) with samples from fractions 15–19 (chitosanase and laminarinase); lane 3: as lane 2, but with the sample containing lipase activity taken from fraction number 10.

lus strains which have a characteristic profile of cell wall-degrading enzymes of glucanolytic and proteolytic enzymes.

Reported experimental data strongly support that mycolytic activity and antagonism are interrelated [7,10,20]. Podile and Prakash [10] showed that extracellular proteins from mycolytic *B. subtilis* AF1 culture exert a significant growth-retarding effect on *Aspergillus niger*. The mycolytic strain suppressed fungal growth by 56–90% in a dual culture. Moreover, *B. subtilis* reduced the incidence of crown rot in a *A. niger*-infected soil.

From the studies reported in the literature, it is evident that hydrolytic enzymes produced by *Bacillus* sp. X-b are involved in its fungal antagonism. Preferential induction of the extracellular mycolytic system of *Bacillus* sp. X-b by biomass and cell walls of certain fungal species, makes it evident that the secreted hydrolases are involved in the dynamics of its antagonism. Natural fungal substrates induced the enzyme activities usually better than colloidal chitin (Table 1) implying that the induction is related to specific recognition mechanisms and could be highly target specific.

Individual enzyme activities involved in the antagonism can be exploited as indicators in microbial screening to assess the antagonistic potential of a strain. Certain details within such indicator enzymes may appear unique and therefore necessitate knowing their exact properties in pure form. Individual activities may be induced differently depending on the inducer and the spectrum of the enzyme activities will reflect specificity of the strain.

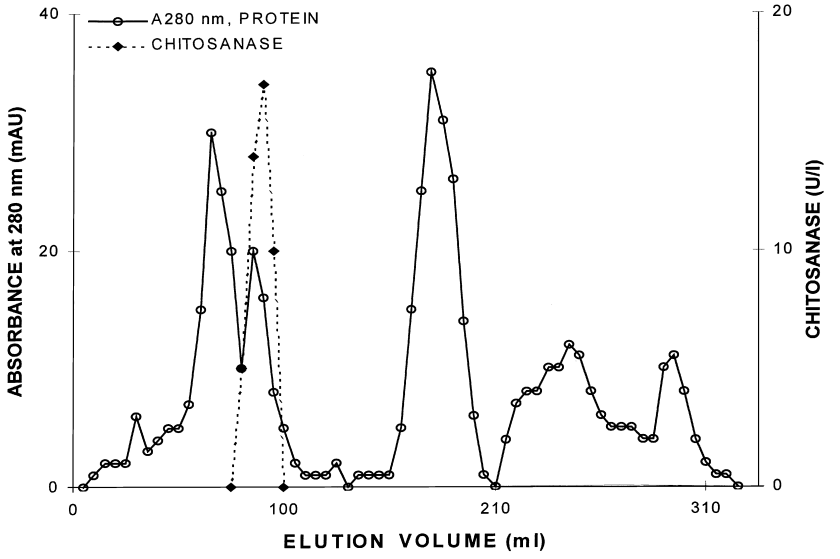

Fig. 7. Gel chromatography on a column of Bio-Gel P-100 of chitosanase produced by *Bacillus* sp. X-b. For conditions, see Materials and methods.

Chitosanase and lipase were purified here to homogeneity as judged by SDS–PAGE. Chitinase was purified 36-fold but it showed two protein bands on SDS–PAGE with M_r 35 000 and 46 000. Laminarinase activity distributed between different fractions on DEAE-chromatography. *Bacillus* sp. X-b seems to produce several chitinases and β-glucanases, including enzymes with different substrate specificity. Such character is common for many of other *Bacillus* strains [13,14,21]. Laminarinase eluted as a sharp band in the DEAE-chromatography of crude preparation, but all attempts of further separation of its different fractions failed because of the loss of activity. Proteolytic activity did not resolve as a single fraction probably because of the existence many of isozymes.

References

[1] B.L. Bertagnolli, F.K. Dal Soglio, J.B. Sinclair, Physiol. Mol. Plant Pathol. 48 (1996) 145.
[2] A. Bruce, U. Srinivasan, H.J. Staines, T.L. Highley, Int. J. Biodeterior. Biodegrad. 97 (1995) 337.
[3] J. Inbar, I. Chet, Soil Biol. Biochem. 23 (1991) 973.
[4] H.-S. Lim, Y.-S. Kim, S.-D. Kim, Appl. Environ. Microbiol. 57 (1991) 510.
[5] M. Lorito, C. Peterbauer, C.K. Hayes, G.E. Harman, Microbiology 140 (1994) 623.
[6] A. Ordentlich, Y. Elad, I. Chet, Phytopathology 78 (1988) 84.
[7] R. Watanabe, N. Ogasawara, H. Tanaka, T. Uchiyama, Agric. Biol. Chem. 52 (1988) 895.
[8] M. Lorito, G.E. Harman, C.K. Hayes, R.M. Broadway, A. Tronsmo, S.L. Woo, A. Di Pietro, Phytopathology 803 (1993) 302.
[9] C. Dass, A. Teyegaga, Aust. J. Bot. 44 (1996) 705.
[10] A.R. Podile, A.P. Prakash, Can. J. Microbiol. 42 (1996) 533.
[11] K.A. Seifert, W.E. Hamilton, C. Breuil, M. Best, Can. J. Microbiol. 33 (1987) 1102.
[12] P. Nielsen, J. Sørensen, FEMS Microbiol. Ecol. 22 (1997) 183.
[13] T. Takayanagi, K. Ajisaka, Y. Takiguchi, K. Shimahara, Biochim. Biophys. Acta 1078 (1991) 404.
[14] M. Yabuki, A. Uchiyama, K. Suzuki, A. Ando, T. Fuji, J. Gen. Appl. Microbiol. 34 (1988) 255.
[15] R. Rodriguez-Kabana, G. Godoy, G. Morgan-Jones, R.A. Shelby, Plant Soil 75 (1983) 95.
[16] T. Imoto, K. Yagishita, Agric. Biol. Chem. 35 (1971) 1154.
[17] T. Vorderwulbecke, K. Kieslih, H. Erdman, Enzyme Microb. Technol. 14 (1992) 631.
[18] D. Haab, K. Hagspiel, K. Szakmary, C.P. Kubicek, J. Biotechnol. 16 (1990) 187.
[19] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[20] K. Horikoshi, S. Iida, Nature 181 (1958) 917.
[21] H. Tanaka, T. Watanabe, J. Ind. Microbiol. 14 (1995) 478.
[22] S. Pleban, F. Ingel, I. Chet, Eur. J. Plant Pathol. 101 (1995) 665.