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Mario Pedraza-Reyes · Félix Gutiérrez-Corona

The bifunctional enzyme chitosanase-cellulase produced by the gram-negative microorganism *Myxobacter* sp. AL-1 is highly similar to *Bacillus subtilis* endoglucanases

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Abstract The gram-negative bacterium *Myxobacter* sp. AL-1 produces chitosanase-cellulase activity that is maximally excreted during the stationary phase of growth. Carboxymethylcellulase zymogram analysis revealed that the enzymatic activity was correlated with two bands of 32 and 35 kDa. Ion-exchange-chromatography-enriched preparations of the 32-kDa enzyme were capable of degrading the cellulose fluorescent derivatives 4-methylumbelliferyl-β-D-cellobioside and 4-methylumbelliferyl-β-D-cellotrioside. These enzymatic preparations also showed a greater capacity at 70° C than at 42° C to degrade chitosan oligomers of a minimum size of six units. Conversely, the β-1,4 glucanolytic activity was more efficient at attacking carboxymethylcellulose and methylumbelliferyl-cellotrioside at 42° C than at 70° C. The 32-kDa enzyme was purified more than 800-fold to apparent homogeneity by a combination of ion-exchange and molecular-exclusion chromatography. Amino-terminal sequencing indicated that mature chitosanase-cellulase shares more than 70% identity with endocellulases produced by strains DLG, PAP115, and 168 of the gram-positive microorganism *Bacillus subtilis*.

Key words β-1,4 Glucanase · Endocellulase · Chitosanase · *Myxobacter*

Introduction

Several microorganisms produce a wide spectrum of enzymes with the ability to degrade polysaccharides linked by β-1,4 glycosidic linkages, e.g., cellulose, chitin, and chitosan. Depending on the mode of attacking the polymer, these enzymes can be classified as endoglycohydrolases or exoglycohydrolases. True cellulolytic microorganisms such as *Trichoderma reesei* produce an enzymatic battery capable of synergistically attacking and degrading crystalline cellulose (Beguin 1990; Saloheimo et al. 1994). In most cases, prokaryotic β-1,4 glucanases catalyze the breakdown of glycosidic linkages of only soluble forms of cellulose such as carboxymethylcellulose and hydroxyethylcellulose (Baird et al. 1990; Beguin 1990). Exocellular microbial β-glucanases are of interest due to their potential application in the brewing industry (Enari and Markkanen 1975), in the bioconversion of agricultural waste material to useful products (Ryu and Mandels 1980), and in the biological control of fungal plant pathogens (Mauch et al. 1988; Grenier et al. 1993).

Genes encoding cellulolytic enzymes have been cloned from different organisms (Henrissat et al. 1989; Gilkes et al. 1991; Henrissat and Bairoch 1993). Based on structural similarities, cellulases and other glycohydrolases have been grouped into several families (Henrissat et al. 1989; Gilkes et al. 1991). *Myxobacter* sp. AL-1 is a gram-negative bacterium isolated from soil; it produces two peptidases (Ensign and Wolfe 1965; Wingard et al. 1972) and a β-1,4 glucanase capable of degrading carboxymethylcellulose and fully deacetylated chitosan (Hedges and Wolfe 1974). The chitosanolytic property of this enzyme has been exploited to produce protoplasts from Zygomycete fungi using partially purified extracts of the excreted enzyme (Genthner and Borgia 1978; Torres-Guzmán et al. 1994).

We report here the study of the synthesis of chitosanase-cellulase activity during the growth cycle of *Myxobacter* sp. AL-1 and the establishment of a protocol for its purification based on cation-exchange and molecular-exclusion chromatography. Purified chitosanase-cellulase was characterized with regard to its capacity for degrading oligomeric forms of cellulose and chitosan. Amino-terminal microsequencing revealed that the mature enzyme possesses more than 70% identity with endocellulases from different *B. subtilis* strains.

M. Pedraza-Reyes (✉) · F. Gutiérrez-Corona
Instituto de Investigación en Biología Experimental,
Facultad de Química, Universidad de Guanajuato,
Apdo. Postal 187, Guanajuato 36000, Guanajuato, Mexico
Tel. +52-473-24996; Fax +52-473-24302
e-mail: pedrama@quijote.ugto.mx

Materials and methods

Organism and growth conditions

The strain used in this study, *Myxobacter* sp. AL-1, was isolated by Ensign and Wolfe (1965) and was kindly provided by P. Sypherd (University of Arizona, USA). The medium used contained 1% (w/v) yeast extract solidified by the addition of 2% (w/v) agar. The kinetics of enzyme production was followed in liquid cultures shaken at 37° C. Culture samples were taken at various times during growth and stored at –20° C until they were processed for enzymatic assay. Growth of the cultures was monitored by measuring optical density at 600 nm.

Enzyme and protein assays

Cellulase activity was measured at 42° C using carboxymethylcellulose as a substrate following a protocol similar to that reported by Hedges and Wolfe (1974). Reducing sugars formed during the assay were detected by the method of Park and Johnson (1949) with the modifications of Ghuysen et al. (1966). Units of enzyme activity were as those reported by Hedges and Wolfe (1974). Specific activity is expressed as units per mg of protein. Enzymatic activity against 4-methylumbelliferyl-β-D-cellobioside, 4-methylumbelliferyl-β-D-cellotrioside, and 4-methylumbelliferyl-β-D-N,N-diacetylchitobioside was assayed essentially as described by Kuranda and Robbins (1987). The chitosanolytic activity of the chitosanase-cellulase enzyme was tested against chitosan oligomers of 2, 3, 4, and 6 units by measuring the release of reducing sugars according to the procedure described by Hedges and Wolfe (1974). Units and specific activity were as those described by Hedges and Wolfe (1974). Protein was measured by the method of Lowry et al. (1951). Carboxymethylcellulose and 4-methylumbelliferyl derivatives were purchased from Sigma (St. Louis Mo., USA). Chitosan oligomers were obtained from Seikagaku America (Rockville, Md., USA).

Purification of chitosanase-cellulase

Chitosanase-cellulase was purified from the supernatant of a 2-1 culture grown for 20 h at 37° C. All the steps of the purification process were carried out at 4° C. After elimination of the cells by centrifugation (17,700 × *g*, 10 min), the proteins secreted to the culture medium were precipitated with $ZnCl_2$ essentially as described by Hedges and Wolfe (1974). The precipitate was dissolved in 25 mM acetate buffer (pH 5.0) and loaded onto a 50 ml CM-BioGel-A column previously equilibrated with the same buffer. The column was washed with 25 mM acetate buffer (pH 5.0) and eluted with 250 ml of a 0.0–0.2 M NaCl linear gradient in the same buffer. Fractions of 5 ml each were collected, and those containing carboxymethylcellulase activity were pooled, freeze-dried, resuspended in 25 mM acetate buffer (pH 5.0) and layered onto a BioGel P-60 column 117 ± 1.7 cm previously equilibrated with the same buffer. The column was eluted with the same buffer, and samples of 3 ml each were collected; fractions containing enzyme activity were pooled and stored at –20° C.

SDS-PAGE and in situ detection of cellulase activity

Electrophoresis in SDS-polyacrylamide gels was carried out as previously described by Laemmli (1970); gels were stained with Coomassie Blue-R250. To establish zymograms of cellulase activity, the separating portions of 10% SDS-polyacrylamide gels were polymerized with 0.1% (w/v) carboxymethylcellulose. Protein samples employed for zymograms were resuspended in 125 mM Tris-HCl (pH 6.8) loading buffer that contained (w/v) 15% sucrose, 2.5% SDS, and 0.02% bromophenol blue; the resuspended samples were boiled 5 min before loading onto the gel. Renaturation of proteins following electrophoresis was performed by incubating the gels in 0.1 M sodium acetate buffer containing 1% (v/v) deionized Triton X-100 for 12 h at 45° C (Trudel and Asselin 1989). To identify protein bands with cellulase activity, the gels were stained with 0.2% (w/v) Congo red and processed as previously reported by Beguin (1983). Alternatively, after renaturation with Triton X-100, gels were stained with Coomassie Blue R-250, photographed, destained with methanol:acetic acid (40:10, v/v), and processed for Congo red staining as indicated above. Zymograms of cellulase activity using methylumbelliferyl-cellotrioside as a substrate were performed as previously described by Van Tilbeurgh et al. (1986) except that the fluorogenic substrate was used at a concentration of 0.1 mM.

Amino-terminal sequencing of chitosanase-cellulase

Chitosanase-cellulase (20 g) partially purified by ion-exchange chromatography was subjected to electrophoresis in a 10% SDS-polyacrylamide gel. Proteins were electroblotted to a polyvinylidene difluoride (PVDF) membrane according to the conditions reported by LeGendre and Matsudaira (1989) and were revealed by staining the membrane with Coomassie Blue R-250. The protein band possessing carboxymethylcellulase activity (see below) was excised from the membrane and processed for amino-terminal sequencing (LeGendre and Matsudaira 1989).

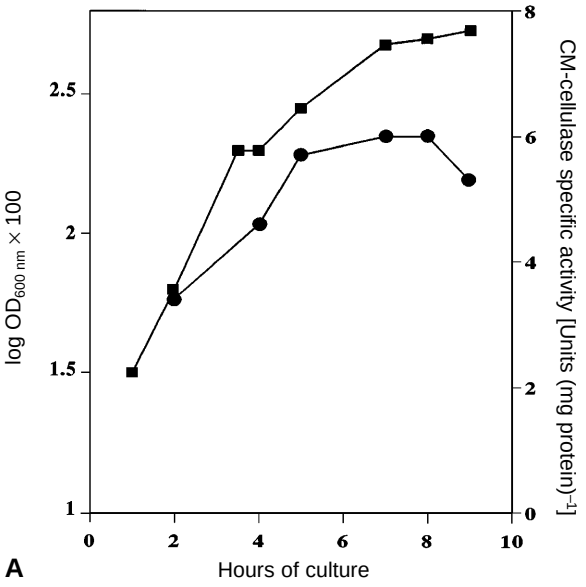
Results

Expression of chitosanase-cellulase during *Myxobacter* sp. AL-1 growth

The temporal appearance of chitosanase-cellulase was followed in liquid culture during *Myxobacter* sp. AL-1 growth (Fig. 1A). As shown, β-1,4 glucanolytic activity appeared early in the exponential phase of growth (2 h) and peaked at 7–8 h, well beyond the onset of the stationary phase. A 20-h culture sample still retained 80% of the activity showed by the 7-h sample (not shown). The proteins secreted into the culture medium were separated by SDS-PAGE, and chitosanase-cellulase was detected by activity staining (Fig. 1B). Two major bands of approximately 32 and 35 kDa were observed in cultures from both the exponential and the stationary phases; however, the 35-kDa band disappeared after 8 h of growth.

Purification of chitosanase-cellulase from *Myxobacter* sp. AL-1

The first purification step of chitosanase-cellulase was carried out by salting out the enzyme activity with $ZnCl_2$, and the precipitate was subjected to further purification by sequential steps of ion-exchange and molecular-exclusion chromatography. The cellulolytic activity was bound to the CM-BioGel-A support and was eluted with 75 mM NaCl (data not shown). Samples of enzyme activity from the ion-exchange column were used for zymogram analysis on SDS-polyacrylamide gels containing carboxymethylcellulose. As shown, this fraction contains three protein bands (Fig. 2, lane 3); however, only the 32-kDa band possessed cellulase activity (Fig. 3A). Following a



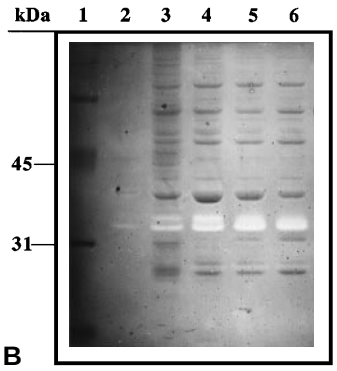


Fig. 1A Temporal expression of chitosanase-cellulase activity (●) during *Myxobacter* sp. AL-1 growth at 37° C (■) in liquid medium. **B** Carboxymethylcellulose (CM-cellulase) activity following SDS-PAGE of proteins secreted into the culture medium during growth of *Myxobacter* sp. AL-1 at 37° C. Culture samples (1.5 ml each) collected during different periods of *Myxobacter* sp. AL-1 growth were defrosted and centrifuged, and the supernatant was precipitated with 10% (w/v) trichloroacetic acid. Protein pellets were suspended in loading buffer and separated in a 10% SDS-polyacrylamide gel containing 1% (w/v) carboxymethylcellulose. The gel was processed for renaturation of proteins as described in Materials and methods and was subsequently stained with Coomassie Blue R-250. After destaining with a solution of methanol:acetic acid (10:40, v/v), the gel was processed for establishing zymograms of carboxymethylcellulase activity as described in Materials and methods. *Lane 1* molecular weight markers, *lanes 2–6* culture samples collected at 2, 5, 7, 8, and 9 h of growth

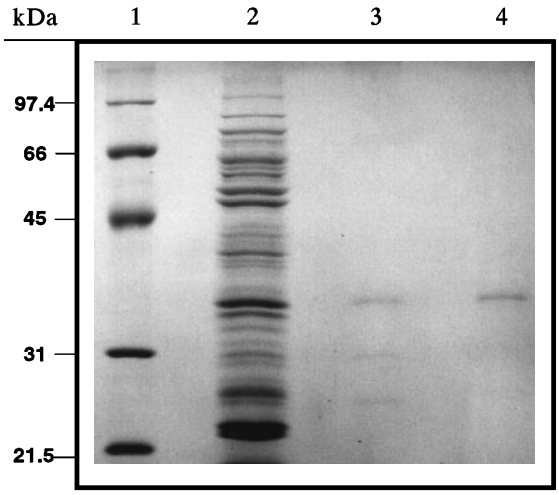


Fig. 2 SDS-PAGE analysis of the different stages of chitosanase-cellulase purification. *Lane 1* molecular weight markers, *2* $ZnCl_2$ crude extract, *3* pool of active fractions from ion-exchange column, and *4* pool of active fractions from BioGel P-60

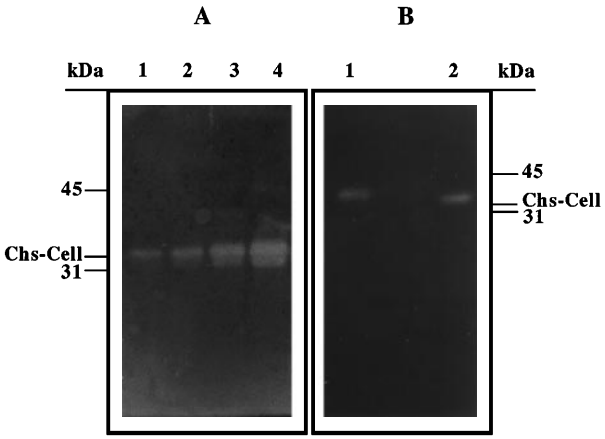


Fig. 3A, B Zymograms of cellulase activity. **A** Various protein amounts of the pool of active fractions obtained from the ion-exchange column were precipitated with 10% (w/v) trichloroacetic acid; the protein pellets were extensively washed and resuspended in loading buffer. Protein samples were subjected to SDS-PAGE, and the gel was processed for carboxymethylcellulase activity as described in Materials and methods. *Lanes 1–4* 10, 20, 40, and 80 μg of protein, respectively. **B** Protein samples from the $ZnCl_2$ crude extract (*lane 1*) and the pool of fractions with enzyme activity from the ion-exchange column (*lane 2*) were precipitated with 10% (w/v) trichloroacetic acid as described above and were further subjected to SDS-PAGE in a 13% polyacrylamide mini-gel. The gel was processed for renaturation, stained, and later processed for activity against 4-methylumbelliferyl-β-D-cellotrioside as described in Materials and methods. *Lane 1* 560 μg protein, *lane 2* 30 g protein (*Chs-Cell* chitosanase-cellulase)

similar approach, it was tested whether the 32-kDa protein band was capable of hydrolyzing in situ the fluorogenic cellulose derivative methylumbelliferyl-cellotrioside. The zymogram shown in Fig. 3B indicates that the protein band of 32 kDa present in both the $ZnCl_2$ extract and the ion-exchange fraction catalyzed the hydrolysis of the fluorescent compound. The β-1,4 glucanase activity recovered from CM-BioGel-A was subjected to further purification on a BioGel P-60 column. The enzyme was applied to the gel and eluted as a single peak of activity (data not shown). The protein profile showed the existence of a single peak that paralleled the peak of enzyme activity. Table 1 shows that the combination of the two types of chromatography led to an approximately 850-fold purification of the chitosanase-cellulase enzyme with a recovery of 6%. SDS-PAGE analysis of the active frac-

Table 1 Purification of chitosanase-cellulase from *Myxobacter* sp. AL-1

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Culture broth	7,269	1.02×10^6	138	1	100
$ZnCl_2$ precipitate	1,056	0.49 ± 10^6	368	3.3	48.9
CM-BioGel-A	7.68	0.11 ± 10^6	14.5×10^3	104	11
BioGel P-60	0.38	4.6×10^4	12.1×10^4	872	4.6

Table 2 Chitosanase-cellulase activity against different substrates at 42 and 70° C

Substrate	Temperature of incubation	
	42° C	70° C
Carboxymethylcellulose	48	11
4-Methylumbelliferyl-β-D-cellotrioside	15.3	0.38
Chitosan 6-mer	7.5	61

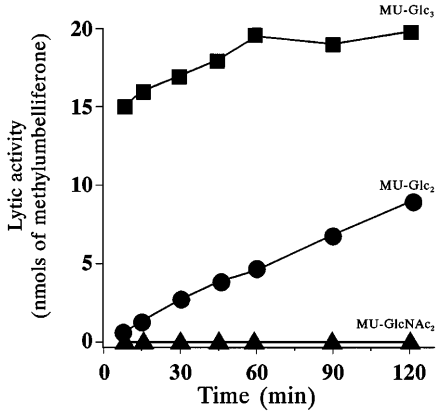


Fig. 4 Hydrolysis of (■) 4-methylumbelliferyl-β-D-cellotrioside (MU-Glc_3), (●) 4-methylumbelliferyl-β-D-cellobioside (MU-Glc_2), and (▲) methylumbelliferyl diacetylchitobioside (MU-$GlcNAc_2$) by the cellulase extract obtained from the ion-exchange column at various time intervals. Reaction conditions and activity determinations were carried out as described in Materials and methods

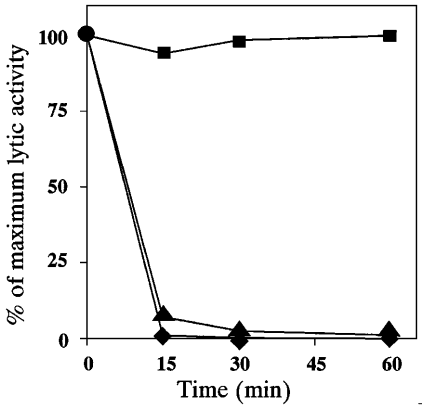


Fig. 5 Heat inactivation of chitosanase-cellulase. Three different samples of chitosanase-cellulase purified by ion-exchange chromatography were incubated at (■) 42° C, (▲) 55° C, or (◆) 70° C; duplicate aliquots (20 µl each) were removed from each sample at 15, 30, and 60 min and processed for cellulase activity using 4-methylumbelliferyl-β-D-cellotrioside as a substrate. The 100% value of cellulase activity (●) was obtained by performing the cellulase assay for 60 min at 42° C

tions recovered in each step of the purification protocol is shown in Fig. 2. The use of ion-exchange chromatography allowed the elimination of most of the protein present in the $ZnCl_2$ precipitate (Fig. 2, lanes 2 and 3) since after this purification step, the active fractions contained only three protein bands with apparent molecular masses of 32, 28, and 25 kDa, respectively (Fig. 2, lane 3). The subsequent use of the BioGel P-60 column resulted in the purification of the enzyme since only the 32-kDa protein band was observed (Fig. 2, lane 4). This finding suggests that chitosanase-cellulase functions as a 32-kDa monomer.

Substrate specificity of chitosanase-cellulase

We investigated the ability of chitosanase-cellulase to degrade methylumbelliferyl-cellobioside and -cellotrioside derivatives (Van Tilbeurgh et al. 1982; Van Tilbeurgh and Claeyssens 1985). Due to the low stability shown by the cellulase activity present in the BioGel P-60 fraction (data not shown), assays of enzyme activity against the fluorescent derivatives methylumbelliferyl-cellobioside and -cellotrioside were carried out with the enzyme purified by ion-exchange chromatography. Figure 4 shows that the enzyme present in the ion-exchange fraction was capable of degrading not only the cellotrioside derivative but also the cellobioside, although the former was a better substrate for the enzyme. The catalytic specificity of the ion-exchange enzymatic fraction was tested for its ability to degrade the chitinase substrate methylumbelliferyl diacetylchitobioside; results plotted in Fig. 4 demonstrate that the chitin derivative is not a substrate for this enzyme. Although chitosanase-cellulase has been reported to have lytic activity against fully deacetylated chitosan (Hedges and Wolfe 1974), the shortest chitosan oligomer recognized as a substrate by the enzyme has not been previously investigated. To this end, the enzyme present in the ion-exchange fraction was incubated with chitosan oligomers of either two, three, four, or six units, and the reactions were carried out at either 42 or 70° C. The results indicated that chitosanase-cellulase degraded only the six-unit chitosan oligomer. However, as shown in Table 2, the enzyme attacked this substrate more efficiently when the reaction was carried out at 70° C. These findings are consistent with the observations that the optimal temperature of chitosanase-cellulase for fully degrading deacetylated polymeric chitosan is 70° C (Hedges and Wolfe 1974). We also tested activity against carboxymethylcellulose and methylumbelliferyl-cellotrioside at either 42 or 70° C. The results demonstrated that the enzyme degraded the cellulose substrates more efficiently at 42° C (Table 2). It was observed that, at either tempera-

Fig. 6 Comparison of the amino-terminal sequences of *Myxobacter* sp. AL-1 chitosanase-cellulase and *Bacillus subtilis* endoglucanases from strains PAP115 (McKay et al. 1986), DLG (Robson and Chambliss 1986), and 168 (Wolf et al. 1995). The N-amino terminal sequence of mature endoglucanases from the three *B. subtilis* strains is identical in this region. *Solid lines* above the *B. subtilis* sequence and below the *Myxobacter* sequence denote the number of residues obtained by amino-terminal sequencing. Identical residues are indicated by *vertical lines*. Conserved changes are indicated by *dots*

ture, the enzyme was incapable of hydrolyzing partially acetylated polymeric chitosan (data not shown). Despite the fact that chitosanase-cellulase attacked the chitosan 6-mer more efficiently when the reaction was carried out at 70° C (Table 2), the enzyme was inactivated by heat (Fig. 5). Although the enzyme was stable at 42° C for at least 1 h, it lost more than 90% activity toward methylumbelliferyl-cellotrioside when it was incubated at either 55 or 70° C for the same time (Fig. 5). As a first approach toward investigating whether chitosanase-cellulase possesses different catalytic sites to degrade two related but different substrates, we tested the ability of the chitosan 6-mer to compete with the enzymatic hydrolysis of the cellulose derivative methylumbelliferyl-cellotrioside. The chitosan 6-mer present at concentrations 400-fold higher (i.e., 1.6 mM) than the concentration of methylumbelliferyl-cellotrioside (0.004 mM) did not cause detectable inhibition of enzyme activity.

Amino-terminal sequence of chitosanase-cellulase

In order to determine the amino-terminal sequence of mature chitosanase-cellulase, samples of the enzyme purified by ion-exchange chromatography were separated by SDS-PAGE and electroblotted to a PVDF membrane; then the protein band possessing enzymatic activity (i.e., the 32-kDa band) was subjected to microsequencing as described by LeGendre and Matsudaira (1989). Figure 6 shows the 25 amino acids of the N-terminus of mature chitosanase-cellulase; at least in this region the enzyme showed 76% identity to endocellulases produced by strains 168, PAP115, and DLG of *B. subtilis* (Wolf et al. 1995; McKay et al. 1986; Robson and Chambliss 1986). Nineteen out of twenty-five residues of chitosanase-cellulase were identical to those of *B. subtilis* strains; three others had conservative changes, i.e., Ser for Thr, Thr for Ser, and Leu for lle (Fig. 6). Taking into account this result, it is reasonable to predict that chitosanase-cellulase might have sequence similarity with other cellulases that are grouped together with *B. subtilis* endocellulases into Family 5 of the classification by Henrissat and Bairoch (1993). Interestingly, the identity of the first amino acid present in the N-terminus of the mature form of chitosanase-cellulase was Ala, a residue almost invariably found in secreted proteins of the *Bacillus* genus (Simonen and Palva 1993).

Discussion

Myxobacter sp. AL-1, a gram-negative microbe, secretes into the culture medium both proteolytic (Ensign and Wolfe 1965; Wingard et al. 1972) and chitosanase-cellulase activities (Hedges and Wolfe 1974). This battery of enzymes (and perhaps others not yet identified) allows this microorganism not only to scavenge organic material waste, but also to attack other bacteria (Ensign and Wolfe 1965) or fungi. As previously reported by Hedges and Wolfe (1974), a single 31-kDa polypeptide contains the cellulase and the chitosanase activities of chitosanase-cellulase. Thus, we used this information to analyze the synthesis of this enzyme during the cell cycle of *Myxobacter* sp. AL-1 by using two criteria: (1) assaying cellulase activity against carboxymethylcellulose and (2) performing zymogram analysis specifically to detect the polypetide(s) possessing this activity. The results of this analysis showed that *Myxobacter* sp. AL-1 produces carboxymethylcellulase activity starting in the exponential phase, although maximum activity was not reached until the stationary phase of growth (Fig. 1A). Activity gel analysis revealed that contribution to cellulolytic activity could be ascribed to two polypeptides having molecular masses of 32 and 35 kDa respectively. The appearance of the 35-kDa band was transitory since it disappeared after 8 h of growth; on the other hand, the 32-kDa band remained even after 20 h of growth. These observations could suggest a precursor-product relationship among these activities, probably by proteolytic processing of the 35-kDa cellulase.

The protocol reported here for purifying chitosanase-cellulase improved the parameters of purification and that enzyme activity recovery six- and three-fold, respectively, as compared to the method reported by Hedges and Wolfe (1974).

The experiments carried out to test the ability of chitosanase-cellulase to degrade methylumbelliferyl-cellotrioside and methylumbelliferyl-cellobioside revealed that this enzyme catalyzed the breakdown not only of the cellotrioside, but also of the cellobioside derivative, although the enzyme catalyzed the hydrolysis of the former more efficiently. This result most probably reflects the preference of chitosanase-cellulase for hydrolyzing cellulose substrates of longer chains and is in agreement with previous results of Hedges and Wolfe (1974), who have demonstrated that the shortest cellodextrin to be attacked by the enzyme is a cellotrioside. Previous results of Van Tilbeurgh et al. (1982) had demonstrated that a *Trichoderma reesei* endocellulase was incapable of hydrolyzing methylumbelliferyl-cellobioside and that this enzyme required at least three units of sugar in the fluorescent glycoside to catalyze its hydrolysis. On the other hand, analysis of the hydrolysis of chitosan oligomers showed that chitosanase-cellulase recognizes as a substrate a minimum size of six units of glucosamine in the chitosan chain, interestingly this substrate was much better degraded at 70° C than at 42° C. In view of the fact that our results demonstrated a lack of thermostability of chitosanase-cellulase

when it was incubated at either 55 or 70° C (Fig. 5), these intriguing results could be reconciled with previous results of Hedges and Wolfe (1974), who have observed that the optimal temperature of chitosanase-cellulase for fully degrading deacetylated chitosan is 70° C, suggesting that this behavior obeys a protective effect exerted by chitosan on the enzyme. On the other hand, chitosanase-cellulase hydrolyzed the cellulose substrates methylumbelliferyl-cellotrioside and carboxymethylcellulose more efficiently at 42° C than at 70° C (Table 2). Taking into account the differential behavior of chitosanase-cellulase in attacking β-1,4 linked polysaccharides of different chemical structures, it could be speculated that this enzyme possesses different catalytic sites for attacking the glycosidic linkages of those polysaccharides. Alternatively, an increase in the reaction temperature (i.e., 70° C) might promote the relaxation of the catalytic site for cellulase activity in the enzyme, thus allowing the fitting of a closely related substrate. The incapability of chitosan 6-mer to compete during chitosanase-cellulase hydrolysis of methylumbelliferyl-cellotrioside might support the suggestion that the enzyme possesses different subdomains to catalyze the hydrolysis of two related, but different polysaccharides.

Amino-terminal sequencing of *Myxobacter* sp. AL-1 chitosanase-cellulase and elucidation of the 25 N-terminal amino acids revealed two aspects of the enzyme. (1) The enzyme possesses 76% identity with endocellulases of *B. subtilis* strains (McKay et al. 1986; Robson and Chambliss 1986; Wolf et al. 1995). Thus, it is reasonable to speculate that *Myxobacter* sp. AL-1 chitosanase-cellulase shares some degree of homology with other cellulases that, in conjunction with *B. subtilis* endocellulases, are grouped in Family 5 of the classification established by Henrissat and Bairoch (1993). (2) The first amino acid present in mature chitosanase-cellulase was found to be Ala which is in agreement with that found in mature *B. subtilis* endocellulases (Fig. 6). This result suggests that signal peptide processing during the export of glucanases might occur by similar mechanisms in both kinds of microorganisms. Although differences exist in the signal peptide of exported proteins among gram-positive and gram-negative microorganisms, some features are conserved, namely, a positively charged amino terminus, a stretch of hydrophobic residues, and a polar carboxy portion with a consensus cleavage sequence (Simonen and Palva 1993). In the case of *B. subtilis*, the endocellulase and other exported proteins conserve the consensus cleavage sequence Ala-X-Ala, and the corresponding signal peptidase acts on the carboxy side of the second Ala (Simonen and Palva 1993). Cloning of the gene that encodes chitosanase-cellulase and elucidation of its primary structure will be fundamental for the establishment of the functional and structural relationship existing in its bifunctional product.

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