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Purification and characterization of a chitosanase from *Streptomyces* N174

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Abstract. A highly efficient chitosanase producer, the actinomycete N174, identified by chemotaxonomic methods as belonging to the genus *Streptomyces* was isolated from soil. Chitosanase production by N174 was inducible by chitosan or D-glucosamine. In culture filtrates the chitosanase accounted for 50–60% of total extracellular proteins. The chitosanase was purified by polyacrylic acid precipitation, CM-Sepharose and gel permeation chromatography. The maximum velocity of chitosan degradation was obtained at 65° C when the pH was maintained at 5.5. The enzyme degraded chitosans with a range of acetylation degrees from 1 to 60% but not chitin or CM-cellulose. The enzyme showed an endo-splitting type of activity and the end-product of chitosan degradation contained a mixture of dimers and trimers of D-glucosamine.

Introduction

Interest in chitosans, polymers of β-(1→4)-linked D-glucosamine units with various degrees of *N*-acetylation, is continously increasing due to a large number of potential medical, industrial and agricultural applications (Sandford 1989). Low-molecular-weight or oligomeric chitosans can be obtained by chemical (Bosso et al. 1986) or enzymic (Izume and Ohtakara 1987) depolymerization. They show interesting biological activities as elicitors of pathogenesis-related proteins in higher plants and inhibitors of growth of some phytopathogenic fungi (Kendra and Hadwiger 1984).

The enzymology of chitosan degradation has only recently begun to be understood. A number of enzymes such as chitinases, chitosanases, lysozymes, exo-*N*-acetyl-β-D-glucosaminidases and exo-β-D-glucosaminidases can hydrolyse chitosan more or less efficiently. For each enzyme studied, a strong relationship between the degree of *N*-acetylation of the substrate and the relative enzyme activity was observed. Whereas, chitinase and lysozyme preferentially attack highly *N*-acetylated polymers, chitosanases hydrolyse more efficiently chitosans with a low *N*-acetyl content (Fenton and Eveleigh 1981; Ohtakara et al. 1988; Pelletier and Sygusch 1990; Pelletier et al. 1990; Sashiwa et al. 1990; Nanjo et al. 1990a; b). In partially *N*-acetylated chitosans, chitinases specifically cleave the *N*-acetyl-β-D-glucosaminidic bonds (Ohtakara et al. 1990; Mitsutomi et al. 1990). Chitosanases from *Bacillus circulans* and *Nocardia orientalis* were shown to hydrolyse chitosan to oligosaccharides with a terminal D-glucosamine (Yabuki et al. 1988; Sakai et al. 1991). However, oligosaccharides with terminal *N*-acetyl-β-D-glucosamine were found in total digests produced by the enzyme from *Penicillium islandicum* (Fenton and Eveleigh 1981).

In order to find microorganisms that secrete chitosan-degrading enzymes, which could be used for biochemical and molecular studies as well as for large-scale production of chitosan oligomers, we screened different types of soil. Bacteria belonging to the actinomycete group were retained for further studies, as they are known to be efficient producers of many extracellular enzymes (Peczynska-Czoch and Mordarski 1988). Here we describe the production and the biochemical characterization of the chitosanase from the strain N174. We recently cloned and overexpressed in *Streptomyces lividans* the gene encoding this chitosanase (Fink et al. 1991).

Materials and methods

Microorganism and culture conditions. Strain N174 was isolated from soil using the following procedure: 1 g of soil was added to 100 ml liquid medium containing MS salts without $(NH_4)_2SO_4$

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(Neugebauer et al. 1991) supplemented with 100 mg/l of peptone (Difco, Detroit, Mich., USA) and 3.0 g/l of chitosan flakes (practical grade, Sigma, St. Louis, Mo., USA). After 4–6 days of growth at 30° C, diluted portions of this selection culture were inoculated on Tryptic Soy Agar (Difco) plates. After different periods of growth, individual colonies with actinomycete-like morphology were tested for their ability to solubilize chitosan by transfer on chitosanase detection agar (CDA). CDA was prepared by mixing 15 g agar, 300 ml of 1% chitosan (dissolved in 0.1 M HCl) and 650 ml distilled water containing the following salts: $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 1.0 g; K_2HPO_4, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnCl_2$, 0.001 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $MnCl_2$, 0.005 g. The pH was adjusted to 6.5 with 5 M KOH and distilled water was added up to 1 l. The medium was sterilized for 15 min at 125° C with constant stirring using a bench-top agar sterilizer (New Brunswick Scientific, Edison, N.J., USA), allowing the formation of a fine chitosan precipitate.

Strain N174 was maintained on sporulation medium SLM3 (DeWitt 1985). For chitosanase production, spores collected from an area of 10–15 cm^2 of the sporulation plate were used to inoculate 4 l flasks, each containing 800 ml of MS with 10 g/l of chitosan flakes, 0.2% (v/v) olive oil (Bertrand et al. 1989) and 0.035% antifoam agent (Antifoam A, Sigma). Incubation was for 108 h at 30° C in a rotary shaker (model G25, New Brunswick Scientific) at 250 rpm. Mycelium was removed by filtration through a Schleicher and Schuell no. 410 filter.

Enzyme purification. The culture filtrate (total volume 1.5 l) was cooled to 4° C (this temperature was maintained throughout the purification procedure) and adjusted to pH 4.5 with 5 M acetic acid. Chitosanase was precipitated following a modification of the procedure of Sternberg and Hershberger (1974): a 2% (w/v) solution of polyacrylic acid [average relative molecular mass (M_r) 250,000, Aldrich, Milwaukee, Wis., USA] was added dropwise to a final proportion of 4 mg/mg extracellular proteins. After 30 min of mixing, the precipitate was collected by centrifugation (11,000 *g*; 30 min) and resuspended in 300 ml distilled water: 1 M NaOH was added until the pH increased to 8.5. In order to remove the residual polyacrylic acid, a 1 M calcium acetate solution was added dropwise (final concentration: 35 mM) and the precipitate was removed by centrifugation and discarded. The supernatant was acidified down to pH 5.0 with 1 M acetic acid.

The acidified supernatant was applied to a 1.6×38 cm CM-Sepharose Fast Flow column (Pharmacia LKB, Baie d'Urfé, Québec, Canada) previously equilibrated with buffer A (25 mM Na-acetate buffer, pH 5). The flow rate was 70 ml/h and 5 ml fractions were collected. Unbound protein was washed from the column with 50 ml buffer A, then a linear 300 ml gradient from 0 to 0.6 M NaCl in buffer A was applied. Fractions containing the chitosanase activity were identified by spotting 10 µl of each fraction on CDA plates and incubating for 4 h at 45° C. The active fractions (total volume 45 ml) were pooled and concentrated six times by overnight dialysis against Bio-Gel Concentrator Resin (Bio-Rad, Richmond, Calif., USA).

The concentrated sample was made up to 20% glycerol and applied on a 1.6×98 cm column packed with Bio-Gel A-0.5 m (200–400 mesh; Bio-Rad) equilibrated with buffer A under gravity pressure. The flow rate was 15 ml/h and 2.5 ml fractions were collected. Active fractions were identified as before and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified chitosanase was stored at −20° C after addition of 1 vol. of sterile glycerol. Under these conditions, the enzyme was stable for many months.

Enzyme assays. The chitosanase activity standard assay contained 950 µl of 0.2% chitosan solution in 50 mM acetate buffer, pH 5.5, 1–20 mU of enzyme and water to a final volume of 1.0 ml. Incubation was for 10 min at 37° C. The reaction was terminated by addition of 300 µl reaction mixture to 1.2 ml neocuproine reagent (Dygert et al. 1965). After 15 min incubation at 100° C in a mineral oil bath, chilling under tap water, dilution with 1.5 ml distilled water and centrifugation in a table-top centrifuge (in order to eliminate the chitosan precipitate), soluble reducing sugars were measured spectrophotometrically at 450 nm. One unit (U) of enzyme was defined as the amount that liberated 1 µmol D-glucosamine equivalent in 1 min under the above conditions.

Exo-β-*N*-acetylglucosaminidase was assayed as previously described (Neugebauer et al. 1991) with *p*-nitrophenyl-β-D-*N*-acetylglucosaminide as substrate. Exo-β-D-glucosaminidase was assayed similarly, except that *p*-nitrophenyl-β-D-glucosaminide (Nanjo et al. 1990b) was used as a substrate.

Enzyme substrates. Chitosan (practical grade, C-0792, Sigma) was used for standard chitosanase assays. Its degree of acetylation (d.a.) was 21%. Chitosan A and B (d.a.s of 61% and 54% respectively) were prepared by acetylation of chitosan Sigma with acetic anhydride according to Hirano and Ohe (1975). Chitosan C (d.a. = 43%) and D (d.a. = 34%) were obtained by a thermo-mechano-chemical treatment (Pelletier et al. 1990). Chitosan E (d.a. = 1%) was prepared by deacetylation of chitosan Sigma by the procedure of Domard and Rinaudo (1983). Chitosan F (d.a. = 1%) was from Katakura Chikkarin, Japan, and was a gift of Dr Kusaoke.

All chitosans were prepared as 10 mg/ml stock solutions in 0.25 M acetate buffer, pH 5.5. Chitosans C and D were only partially soluble and enzymic assays were carried out using their soluble fraction.

Glycol chitosan, carboxymethyl cellulose, purified chitin, laminarin were from Sigma. Colloidal chitin was prepared using the procedure of Hsu and Lockwood (1975). Avicell was from FMC (Philadelphia, Pa., USA).

Analytical procedures. Protein concentration was estimated by the method of Stoscheck (1990) with bovine serum albumin (BSA) as standard. The NH_2-terminal protein sequence was determined by Edman degradation with an Applied Biosystems 473A protein sequencer. Thin-layer chromatography (TLC) of end-products of chitosan degradation was performed as described by Neugebauer et al. (1991). The system used for analytical high-performance size-exclusion chromatography consisted of a Waters 590 pump, a WISP 512 automatic injector and a Waters 410 refractive index detector. Two TSK gel columns in series (600 X 7.5 mm) thermostatted at 25° C were employed: G3000PW and G4000PW (Toso Haas, Philadelphia, Pa., USA). The eluent, degassed and filtered (0.45 µm) 2% acetic acid with sodium nitrate (0.2 M) and sodium azide (0.1%), was pumped at 0.5 ml/min. The samples from enzymatic digestions were filtered (0.45 µm) and 50-µl aliquots were injected (in some cases the samples had to be diluted in order to keep the dissolved solids concentration under 4 mg/ml). All the data were acquired and processed with the aid of the Waters Maxima 820 software programme (Millipore Waters, Mississauga, Ontario, Canada).

Chemotaxonomical procedures. The diaminopimelic acid form in the cell wall and the predominant sugar in whole-cell hydrolysates were analysed by TLC according to Staneck and Roberts (1974). The GC content of total DNA was measured by the method of Ulitzur (1972). Mycolic acids were analysed according to Tomiyasu and Yano (1984).

Results

Characterization of the N174 strain at the genus level

The actinomycete N174 was isolated from a slightly acidic soil in a sugar maple grove near Sherbrooke. It was selected as the most active chitosanase producer among 50 other actinomycete isolates. It produces abundant aerial mycelium on Tryptic Soy Agar and sporu-

lates well on SLM3 medium. Spores are formed in long chains. The GC content of its DNA is 68±2.5 mol%. *Meso*-diaminopimelic acid is the predominant form in the cell walls of vegetative mycelium but the LL-form becomes predominant in spores. Galactose is the predominant sugar in whole-cell hydrolysates (arabinose was not detected). According to these data, the N174 strain was first classified in the genus *Kitasatosporia* (Ōmura et al. 1982) and the name *Kitasatosporia* N174 was used in our previous work (Fink et al. 1991). However, recently the whole genus *Kitasatosporia* has been transferred to the genus *Streptomyces* (Wellington et al. 1992). Thus the N174 strain will be referred as *Streptomyces* N174.

Chitosanase production. The best results for chitosanase production were obtained in a medium containing chitosan as the sole carbon source. Various enrichments (0.5% peptone; 0.5% casamino acids; 1% starch) increased the growth rate but decreased chitosanase production to, respectively, 5–10%, 5–10% and 20–50% of the levels obtained with chitosan. 0.5% D-glucosamine as sole carbon source also induced chitosanase production but at levels reaching only 15–25% of those obtained in chitosan medium.

Enzyme purification. Various procedures for the concentration of the enzyme were attempted: precipitation with acetone, ethanol, ammonium sulphate and polyethylene glycol 3350. For unknown reasons, none was satisfactory, resulting in significant loss of enzyme activity. Having established that the N174 chitosanase has a slightly alkaline pI (Fink et al. 1991), polyacrylic acid, a precipitant used in large-scale preparations of some industrial enzymes (Sternberg and Hershberger 1974) was successfully tried. Direct addition of polyacrylic acid solution to the culture supernatant (the pH of which varied from 5.8 to 6.5 in different experiments) gave relatively low enzyme recoveries (25–55%). Acidification of the culture supernatant with 1 M acetic acid (down to pH 4.5) before polyacrylic acid addition resulted in better enzyme recoveries (around 85%). The optimal ratio polyacrylic acid/total extracellular proteins was found to be around 4 (Fig. 1).

After two subsequent chromatographic steps, a chitosanase was obtained, homogeneous as estimated from polyacrylamide gels stained with Coomassie Blue and from NH_2-terminal amino acid sequencing, with a specific activity of 59.8 U/mg protein (Table 1).

The NH_2-terminal sequence of the purified enzyme was determined to be Ala-Gly-Ala-Gly-Leu-Asp-Asp-Pro-His-Lys-Lys-Glu-Ile-Ala-Met-Glu-Leu-. Previously, the M_r of the purified enzyme was estimated to be approximately 29,500 by SDS-PAGE (Fink et al. 1991).

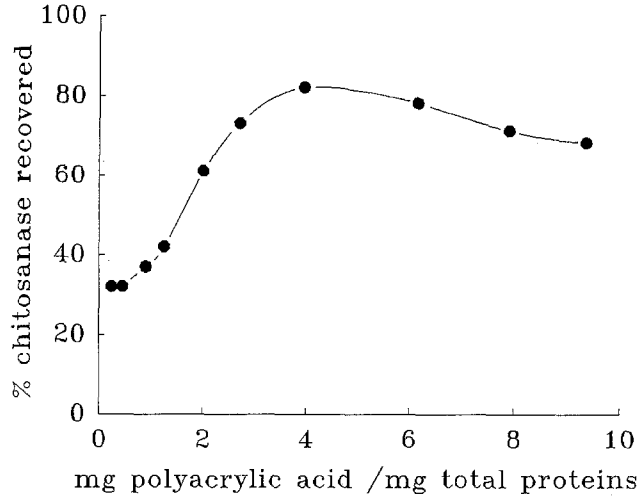

Fig. 1. Precipitation of chitosanase with various levels of polyacrylic acid at pH 4.5

Biochemical properties of the chitosanase

The optimal reaction conditions, enzyme stability and kinetic parameters were determined with Sigma chitosan as the substrate. The pH range for activity extended from 4.0 to 6.0 with a maximum at 5.5 (Fig. 2). A sharp drop in activity was observed at pH values higher than 6.5, coinciding with precipitation of the substrate. The enzyme was stable in a pH range of 4.5 to 6.0 for at least 4 h at 37° C. The optimum temperature for a 10 min reaction at pH 5.5 was about 65° C (Fig. 2).

The apparent K_m determined from a single reciprocal plot (not shown) was 0.088 mg/ml and the V_{max} was 96.5 U/mg. Substrate inhibition was observed at chitosan concentrations higher than 1 mg/ml.

Thermal stability of the chitosanase was determined by incubating the enzyme in 50 mM acetate buffer, pH 5.5, at various temperatures and for various periods of time in the absence of chitosan, after which the residual activity was determined by the standard assay. The enzyme was stable at 37° C but its stability decreased rapidly above 40° C (Fig. 3). As described for chitosanase A from *Bacillus megaterium* P1 (Pelletier and Sygusch 1990), stability could be improved by addition of 0.1 mg/ml of BSA to the preincubation buffer. These stud-

Table 1. Purification procedure for the chitosanase from *Streptomyces* N174

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Acidified culture broth	366	12720	34.7	100	1.00
Polyacrylic acid	232	10040	43.3	79	1.25
CM-Sepharose	122	6105	50.1	48	1.44
Bio-Gel A 0.5 M	57.4	3434	59.8	27	1.72

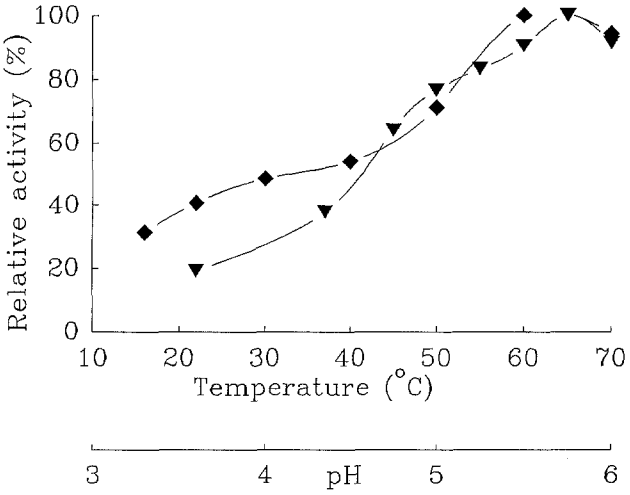

Fig. 2. Effect of temperature (▼) ans pH (◆) on the chitosanase activity. Purified chitosanase [20 mU/ml] was incubated for 10 min with substrate at the temperatures or pH values indicated and reducing sugars were measured by the neocuproine assay

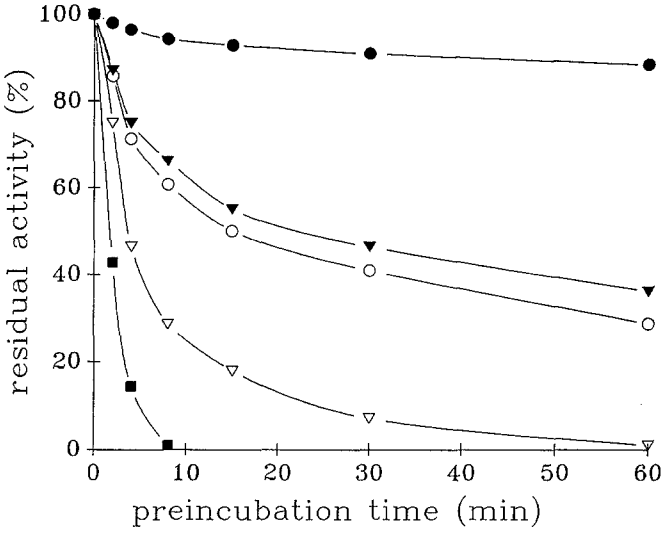

Fig. 3. Effect of temperature on the chitosanase stability. Purified chitosanase (0.28 U/ml) in 50 mM Na-acetate buffer, pH 5.5, was incubated without substrate at various temperatures in the presence or absence of bovine serum albumin (BSA, 0.1 mg/ml). The residual activity was determined at intervals by the standard assay: ○, 45° C, no BSA; ●, 45° C, + BSA; ▽, 50° C, no BSA; ▼, 50° C, + BSA; ■, 55° C, no BSA

ies showed, however, that the enzyme is relatively thermolabile.

Substrate specificity and mechanism of enzyme action

The purified enzyme was specific for chitosan degradation. No hydrolysis of colloidal chitin, purified chitin, CM-cellulose, Avicell, laminarin, *N-N*-diacetylchitobiose, *p*-nitrophenyl-β-D-*N*-acetyl-glucosaminide or *p*-nitrophenyl-β-D-glucosaminide was observed.

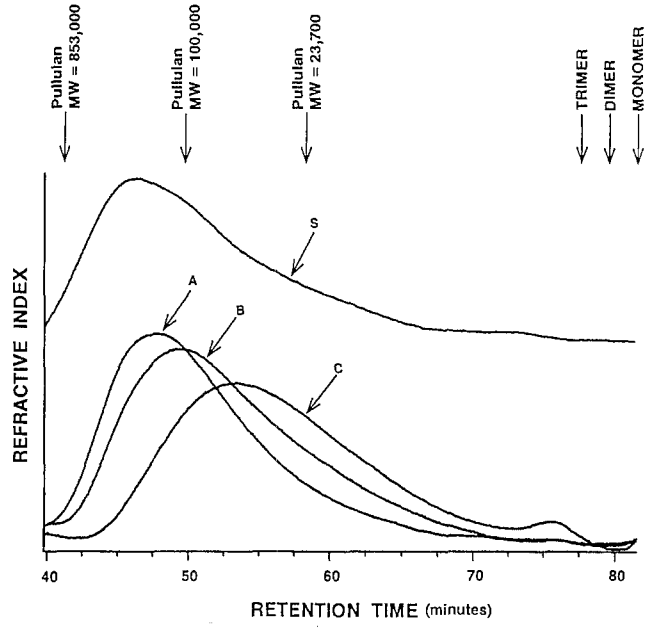

Fig. 4. HPLC analysis of products of partial hydrolysis of Sigma chitosan by purified chitosanase. Enzyme (*A*, 6.25 mU/ml; *B*, 12.5 mU/ml; *C*, 25 mU/ml) was incubated at 37° C in a 1-ml solution containing 2 mg chitosan Sigma in 50 mM Na-acetate buffer, pH 5.5. After 10 min, 50 µl samples were heated to 100° C for 10 min to inactivate the enzyme and were then analysed by high performance size-exclusion chromatography. *S*, undigested chitosan Sigma; MW, relative molecular mass

The relationship between enzyme activity and the degree of acetylation of the chitosan substrate was tested on a series of chitosans prepared by various chemical methods. Maximal rates of hydrolysis was observed for chitosans with low degrees of acetylation (1–21%: chitosans E, F and Sigma) but the N174 chitosanase was able to hydrolyse efficiently chitosans in the range of d.a.s from 34 to 61%, still exhibiting half of the maximal hydrolysis rate against the most acetylated of these substrates, chitosan A (d.a. = 61%). Glycol chitosan was hydrolysed at 35% of the maximal rate.

HPLC analysis of products obtained from the early stages of reaction showed a rapid decrease in the M_r of the chitosan substrate (Fig. 4), indicating that the enzyme hydrolyses chitosan in an endowise manner. The products of a complete digestion of chitosan were analysed by TLC (not shown). As for other chitosanases (Price and Storck 1975; Pelletier and Sygusch 1990) the main products detected were dimers and trimers of D-glucosamine with only traces of free D-glucosamine or higher oligomers. Thus, the tetramer should be the shortest oligomer still recognized as a substrate by the chitosanase.

Discussion

There is a marked heterogeneity in the reported biochemical properties of chitosanases. In early studies, chitosanases were classified in two groups: those specific for chitosan and those able to hydrolyse both chitosan

and CM-cellulose. This classification has however been criticized by some authors (Pelletier and Sygusch 1990). Other studies show that the various chitosanases differ in their isoelectric point, substrate specificities, their requirements for *N*-acetylglucosamine residues for expression of maximal activity and the kind of residue adjacent to the cleavage site on the chitosan chain (Fenton and Eveleigh 1981; Yabuki et al. 1988; Sakai et al. 1991). Most probably, a comprehensive classification of chitosanases could be built only by the comparison of a series of amino acid sequences derived from gene cloning and DNA sequencing. Such work is now in progress in our laboratories.

In many respects, the *Streptomyces* N174 chitosanase, described in this report, resembles that of *Streptomyces* sp. no. 6 (Price and Storck 1975). Both enzymes cannot be precipitated from culture filtrates by conventional laboratory techniques, both exhibit the same mechanism of chitosan hydrolysis, they do not attack chitin and CM-cellulose and they have similar M_rs. The pI of the *Streptomyces* sp. no. 6 chitosanase was not determined, but can be deduced as being near to neutral from its behaviour in ion exchange chromatography, thus it is similar to that of N174 chitosanase. The *Streptomyces* sp. no. 6 chitosanase has however a much higher apparent K_m for chitosan compared with that of N174, suggesting that there are significant differences between the two enzymes.

The strain *Streptomyces* N174 is one of the most efficient producers of chitosanase described so far in the literature. In filtrates obtained after cultivation of N174 in chitosanase production medium, the chitosanase accounts for approximately 50–60% of total extracellular proteins. Precipitation with polyacrylic acid, an easy and inexpensive step, gives enzyme preparations with a chitosanase specific activity reaching 72–75% of that of the purified enzyme. This enzyme preparation is stable for many weeks at 4° C and does not contain other chitino- or chitosano-lytic activities (data not presented). Thus, the N174 strain is suitable for large scale chitosanase production in the range of hundreds of milligrams or of grams). This will permit its use in studies of enzyme immobilization or crystallography.

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