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Chitinases of *Streptomyces kurssanovii*: purification and some properties

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Four major chitinases, with molecular weights of 42, 40, 26 and 20 kDa, were detected in the culture supernatant of *Streptomyces kurssanovii*. They were isolated by means of metal–ion affinity chromatography and hydrophobic-interaction chromatography on butyl-toyopearl 650 M and octyl-sepharose CL-4B with a recovery of 57% of total initial activity. The purified chitinases were homogeneous according to SDS-PAGE. For two enzymes, Chi-42 and Chi-26, molecular weights, pI values, amino acid composition, as well as the influence of pH, temperature and some metal ions on activities and stabilities were determined. The chitinases Chi-42 and Chi-26 hydrolysed chitin, chitosan (85% deacetylated) and CM-chitin (69% substituted) in an endo-splitting manner. The apparent K_m values for Chi-42 calculated from Lineweaver–Burk plots were $0{\cdot}16 \times 10^{-4}$ M (chitosan) and $0{\cdot}18 \times 10^{-4}$ M (CM-chitin) at pH = 4·0 and $t = 37°C$. Both chitinases hydrolysed tetramers and pentamers of *N*-acetylglucosamine, but less readily hydrolysed its trimer. The HPLC analysis of the reaction products indicated that the chitinase Chi-26 also catalysed a transglycosylation reaction, since oligosaccharides with a higher degree of polymerization than the initial substrates were produced.

INTRODUCTION

Application of materials prepared from chitin and its deacetylated derivative, chitosan, is very promising in different fields, including medicine, pharmacology and biochemistry. In many instances, however, transformation of chitin and chitosan into oligomers with enhanced solubility in aqueous media under the action of chitinolytic enzymes seems to be advantageous. Chitooligosaccharides with polymerization degrees of 6–7 are nontoxic substances that display specific anticancer (Suzuki *et al.*, 1986) and antibacterial (Shibasaki *et al.*, 1988) activities. Chitooligosaccharides of lower molecular weights could be useful as antifungal substances in agriculture (Allan *et al.*, 1979).

The aim of this work was to isolate the *Streptomyces kurssanovii* chitinolytic enzymes and examine their activities in the conversion of different substrates into low-molecular-weight chitooligosaccharides.

MATERIALS AND METHODS

Enzymes and substrates

Streptomyces kurssanovii was cultured for 96 h in a 500 ml shaken flask containing 80 ml of a complex medium (Pheniksova *et al.*, 1974) composed of 2% crab shell chitin, 0·2% peptone, 0·1% yeast extract, 0·3% K_2HPO_4 and 0·1% $MgSO_4{\cdot}7H_2O$ (pH 7·5, 30°C). The agitation rate was 300 rev/min. Culture supernatants were obtained by centrifugation for 30 min at a rate of 5000 rev/min.

Deproteinized and demineralized crab shell chitin contained 10% moisture, 2% ash and less than 15% deacetylated amino groups. Crab shell chitosan contained 3·4% moisture, 0·07% protein and 0·13% ash. The chitin and chitosan were obtained from the Institute of Bioorganic Chemistry, Vladivostok, Russia. Colloidal chitin was prepared by the procedure of

Chigaleytchik *et al.* (1976). The degree of deacetylation of the chitosan used, when determined by a spectrophotometric method (Muzzarelli & Rochetti, 1985), appeared to be 85%. The molecular weight of the chitosan was about 200 kDa, determined by the viscosimetric method using Mark–Houwink coefficients proposed by Gamzazade *et al.* (1985).

Carboxymethyl (CM)-chitin was obtained according to Tokura *et al.* (1983). The molecular weight of the CM-chitin was 198 kDa, when determined by the viscosimetric method with Mark–Houwink coefficients proposed by Inone *et al.* (1982). The degree of CM-substitution was 69%.

A series of *N*-acetylchitooligosaccharides $(GlcNAc)_n$ ($n = 3$–5) was prepared as follows. Crab shell chitin was hydrolysed by 1 N HCl under nitrogen at 100°C for 4 h. Reacetylated with Ac_2O chitin oligosaccharides were purified by gel-filtration chromatography on a Bio-gel P 2 column (3·5 cm × 110 cm) in 0·1 M acetic acid. The fraction of low-molecular-weight *N*-acetylchitooligosaccharides was then separated using preparative HPLC on a silica gel column C_{18} (2·0 cm × 30 cm) provided by the Institute of Organic Chemistry of the Academy of Sciences of Russia. *N*-Acetylchitooligosaccharides (100 mg) dissolved in 1500 μl of water were injected into the column and eluted with water. The elution pattern was monitored at 214 nm, characteristic of *N*-acetyl groups. Individual oligomers were collected and lyophilized; the yield was 8–10%.

Chitinase assay

As the standard assay of chitinase, a mixture of colloidal chitin suspended in 1·0 ml of 0·1 M sodium phosphate buffer (pH 7·0) and 0·5 ml of enzyme solution was incubated at 37°C. After 60 min of incubation, the mixture was heated for 5 min in a boiling water bath and centrifuged. The amount of reducing sugars in the supernatant was measured by the reaction with 3,5-dinitrosalysilic acid (Miller, 1959) using *N*-acetylglucosamine as a standard. One unit of chitinase activity (U) was defined as the amount of enzyme that liberated 1 μM of reducing sugar per minute.

Chitobiase assay

Activity of *N*-acetyl-β-D-glucosaminidase (chibotiase) was measured by monitoring the rate of formation of *p*-nitrophenol from *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside. The *p*-nitrophenol released was measured at 405 nm using a molar absorptivity of $18·5 \times 10^6$ cm²/M (Kubicek *et al.*, 1986). One unit of chitobiase activity was defined as the amount of enzyme that catalysed the formation of 1 μM of *p*-nitrophenol in 1 min.

Protease assay

Protease activity was estimated by hydrolysis of casein (Kaversneva, 1971) using L-tyrosine as a standard. One unit of protease activity was expressed as the amount of enzyme that liberated 1 μM of tyrosine per minute.

Effect of pH on activity and stability

The ph optima of chitinases activity were determined in 0·1 M citric acid–0·2 M disodium phosphate buffers in the pH range 3·0–7·3 and in 0·2 M sodium phosphate buffers in the pH range 7·0–9·0. After preincubation at 37°C for 10 min, 1·0 ml of colloidal chitin suspension in buffers of different pH was treated with 0·3 ml of the enzyme solution (0·03 U/ml). After 10 min at 37°C, the reaction was stopped at 100°C and the activity of the chitinase was measured as previously described.

The effect of pH on the enzyme stability was observed by assaying the enzyme activity after incubation at 25°C for 24 h at each of the pH values. The results were expressed in per cent, with respect to the maximal activity and stability of the enzyme.

Effect of temperature on activity and stability

The thermal stability of chitinases was determined by incubating samples of each enzyme (0·03 U/ml) in 0·1 M citric acid–disodium phosphate buffer (pH 4·0) for 60 min at 25, 37, 50, 60 and 70°C. The samples were cooled and then assayed for chitinase activity at 37°C for 60 min with colloidal chitin, as usual. The results were expressed in per cent, with respect to activity of the non-heated control solution of the enzyme.

The activities were measured at the above set of temperatures under standard conditions. The activity of enzymes at 50°C was arbitrarily adopted as 100%.

Effect of metal ions

Each enzyme was incubated at 37°C for 5 min in the presence of 1×10^{-3} M of each of the following metal ions: Ca^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Ag^{+}, Sn^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, as well as in the presence of 10^{-2} M EDTA. The residual activity was measured by the standard method and expressed in per cent, with respect to the activity of the enzyme sample before the treatment.

Assay of the relative rates of hydrolysis of *N*-acetylchitooligosaccharides

The enzyme (0·02 U) was incubated with a 0·5 mM solution of *N*-acetylchitooligosaccharides (degree of polymerization $n = 3$–5) in 0·1 M citric acid–0·2 M disodium phosphate buffer (pH 4·0) at 37°C for 5 and 30 min. The amount of reducing sugars liberated was measured as described above. The rate of hydrolysis of the *N*-acetylchitotetraose was arbitrarily set at 100%.

The products of the hydrolysis reaction were analysed by HPLC. A 20-μl portion of the reaction mixture was injected into a silica gel C_{18} column (10 mm × 180 mm) and eluted with water at a flow rate of 0·5 ml/min. The *N*-acetylchitooligosaccharides eluted were monitored at 214 nm.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical electrophoretic unit using a 10% gel, according to the procedure of Laemmli (1970). Molecular weight markers used were: phosphorylase (94 000 Da), BSA (67 000 Da), egg albumin (43 000 Da), carbonic anhydrase (30 000 Da), trypsin inhibitor from soybean (20 100 Da), α-lactoglobulin (14 400 Da) (Pharmacia). A plot of log of the marker molecular weights against mobility was found to be linear.

Isoelectric focusing was carried out with a flat-bed electrofocusing apparatus (Chiyo Kalur, Estonia) on the thin-layer polyacrylamide gel (Pharmacia) in the pH range from 3·5 to 9·5. The anolyte was 1 M H_3PO_4 and the catolyte was 1 M NaOH. Electrophoresis was continued at a constant voltage (600 V) for 6 h at 4°C. Gels were washed with 3·5% sulphursalysilic acid–10% trichloroacetic acid (w/v) to remove the ampholytes and then stained with 0·005% Coomassie Blue G-250 in methanol–acetic acid–water (10:1:9, by vol.). To determine the pH gradient, gel strips were macerated with 2 ml of distilled and de-ionised water and, after standing for 2 h, the pH values were determined at ambient temperature.

Amino acid analysis

Samples containing 3–5 mg of lyophylized proteins were hydrolysed under N_2 with 5·7 M HCl in the presence of 0·02% β-mercaptoethanol (Merck) and 0·25% (w/v) phenol at 110°C for 24 h. Analysis of samples was carried out with a Beckman 121 M amino acid analyser.

The protein content was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard protein.

Preparation of antibody against chitinases

Rabbit antisera were produced by injecting into rabbits each of the two purified chitinases, Chi-42 and Chi-26. After the first injection of 1 mg protein, three subsequent injections were given at intervals of 4, 7 and 12 days using 0·5 mg of each protein followed by two injections at intervals of 4 weeks. Blood samples (30–40 ml) were collected 2 weeks after the last injection. According to Ouchterlony's (1953) double immunodiffusion in 1% agar gel, the antibodies prepared against either Chi-42 or Chi-26 were diluted with sodium phosphate buffer (pH 6·5) and then applied to the lanes cut alongside the wells containing the enzymes. Diffusion was allowed to proceed overnight in a humid incubator at ambient temperature. The gel was partially dried and washed twice to remove the unprecipitated antibodies and then stained with 0·5% Coomassie Blue R-250 in methanol–acetic acid–water (4:1:4, by vol.). Destaining was done after 30 min with the same mixture of solvents.

Kinetic measurements

The initial rates of hydrolysis of water-soluble chitin derivatives, carboxymethyl (CM)-chitin and chitosan by chitinases were measured using a Ubbelohde-type viscometer at 25°C. According to the method of Rabinovich *et al.* (1977), the expression for the initial rate of destruction of a polymeric substrate under an endo-splitting enzyme action is

$$V_0 = \frac{1}{\alpha M_0 [\eta]_0 \eta_{rel} 7/8} (d\eta_{rel}/dt) \quad (1)$$

where M_0 is the molecular weight; $[\eta]_0$ is the intrinsic viscosity of the initial substrate; α is the exponent in the Mark–Houwink equation; η_{rel} is the relative viscosity at the beginning of the hydrolysis, estimated by extrapolation of the measurable values of relative viscosity to initial time of the reaction; and $d\eta_{rel}/dt$ is the rate of the alteration of η_{rel}.

On the other hand, according to the Michaelis–Menten equation, the initial rate of enzymatic reaction is

$$V_0 = \frac{V_{max}[S]_0}{K_m + [S]_0}. \quad (2)$$

Equations (1) and (2) provided the evaluation of the kinetic parameters K_m and V_{max} in the double-reciprocal Lineweaver–Burk plot. The enzymatic reaction was initiated by addition of the Chi-42 (0·07–0·57 μM) to a solution of polymeric substrate (0·2–0·7 g/dl) in 0·3 M sodium acetate buffer (pH 4·5) at 25°C. Steady-state kinetic analysis was performed on the initial rates data from the Ubbelohde viscometer. The real reaction time was calculated as $t = t_r + t/2$, where t_r was the reaction time and $t/2$ was half the elapse time. The decrease of relative viscosity and rate of alteration of relative viscosity were found from the relative viscosity–reaction time plot.

Purification of chitinases

Chromatography on Cu^{2+}–IDA–agarose

The *S. kurssanovii* culture filtrate containing 100 optical units (o.u.) (280 nm) was loaded onto a Cu^{2+}–IDA–agarose column (2·0 cm × 4·5 cm) equilibrated with 0·1 M sodium acetate (pH 7·5), containing 0·5 M NaCl. The column was washed with 90 ml of the same solution

at 0·8 ml/min. Fractions (3·5 ml each) were assayed for chitinase, chitobiase, protease, protein and pH. Protease and chitobiase were detected in the effluent (peak 1, Fig. 1). The column was then washed with 40 ml of 0·1 M sodium acetate buffer (pH 5·0) and 0·5 M in NaCl, followed by 40 ml 0·1 M sodium acetate buffer (pH 4·0) and 0·5 M in NaCl. Fractions containing chitinase activity (peaks 2 and 4, Fig. 1), were combined, dialysed against water and freeze-dried.

Chromatography on butyl-toyopearl 650 M

The pooled and dried fractions of peak 4 of Fig. 1 (16–20 o.u., 280 nm) were dissolved in the minimal volume of 0·1 M sodium phosphate (pH 7·0) containing 1 M Na_2SO_4. Each enzyme preparation was loaded onto a column of butyl-toyopearl 650 M (2·5 cm × 7·5 cm), pre-equilibrated in the above buffer, and eluted at a flow rate of 0·8 ml per min with a 70-ml linear gradient of 1·0–0 M sodium sulphate in the same buffer. Then, the column was eluted with 0·02 M sodium phosphate buffer (pH 7·0) and water. Fractions of 3·5 ml each were collected and assayed for protein at 280 nm and chitinase activity as described above. Two peaks (5 and 6, Fig. 2), containing chitinase activity, were obtained from peak 4.

Chromatography on octyl-sepharose CL-4B

The concentrated fractions 5 and 6 (Fig. 2) eluted from the butyl-toyopearl column were additionally chromatographed on a Pharmacia octyl-sepharose CL-4B column (2·0 cm × 10·0 cm) equilibrated with 0·1 M sodium phosphate (pH 7·0) containing 1 M Na_2SO_4. The retained proteins were eluted at a flow rate of 0·8 ml/min by a linear salt gradient (1·0–0 M sodium sulphate) in 0·1 M sodium phosphate buffer (pH 7·0). Effluents

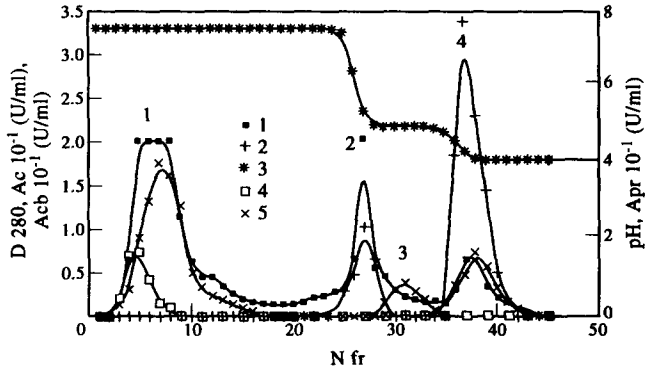

Fig. 1. Metal affinity chromatography on Cu^{2+}–IDA–agarose. The culture filtrate of *S. kurssanovii* (105 o.u., 280 nm) was applied to a column (2·0 cm × 4·5 cm) of affinity adsorbent previously equilibrated with 0·1 M sodium acetate (pH 7·5) + 0·5 M NaCl. After washing the column with the same solution, the enzymes were eluted with 0·1 M sodium acetate buffer (pH 5·0) + 0·5 M NaCl, followed by 0·1 M sodium acetate buffer (pH 4·0) + 0·5 M NaCl. The elution rate was 0·8 ml/min. 1, Absorbance at 280 nm; 2, chitinase activity (U/ml); 3, pH; 4, chitobiase activity (U/ml); 5, protease activity (U/ml).

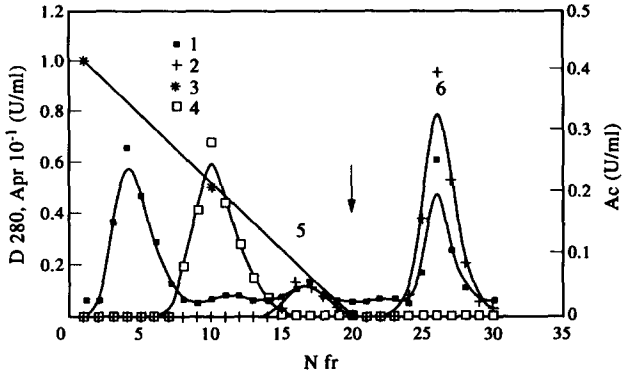

Fig. 2. Separation of fraction 4 (Fig. 1) using butyl-toyopearl 650 M. Enzyme preparation (fraction 4) containing 16 o.u (280 nm) was applied to a column (2·5 cm × 7·5 cm) of butyl-toyopearl 650 M which was equilibrated with 0·1 M sodium phosphate buffer (pH 7·0) containing 1 M Na_2SO_4. Enzymes were eluted with a 70-ml gradient of 1·0–0 M Na_2SO_4 in the same buffer, developing the column with 0·02 M sodium phosphate buffer (pH 7·0). Buffer change is indicated with an arrow. 1, Absorbance at 280 nm; 2, chitinase activity (U/ml); 3, Na_2SO_4 concentration (M); 4, protease activity (U/ml).

were monitored at 280 nm and their chitinase activity was determined. By such a procedure, peaks 7 and 8 (Fig. 3) were obtained from peak 6; also, peak 9 was obtained from peak 5. Peak 10 resulted from peak 2 by the same chromatographic procedures. The pooled fractions were dialysed against water and lyophylized.

Molecular weight determination

Molecular weight determination of native proteins was performed by gel-filtration on Sephacryl S-200

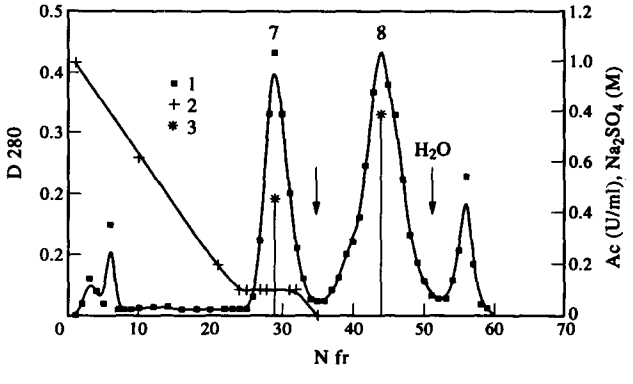

Fig. 3. Separation on octyl-sepharose CL-4B of fraction 6 (Fig. 2). The fraction 6 obtained after chromatography on butyl-toyopearl was dialysed against 0·1 M sodium phosphate buffer (pH 7·0) containing 1·0 M Na_2SO_4 and then applied to an octyl-sepharose column (2·0 cm × 10·0 cm) equilibrated with the same buffer. The first enzyme (fraction 7) was eluted with 0·1 M disodium sulphate in the initial buffer; the second enzyme (fraction 8) was eluted by 0·02 M sodium phosphate buffer (pH 7·0). The column was finally washed with water. Buffer changes are indicated with arrows. 1, Absorption at 280 nm; 2, Na_2SO_4 concentration (M); 3, chitinase activity (U/ml).

(2·0 cm × 60 cm) (Pharmacia), using BSA (67 kDa), ovoalbumin (45 kDa) and papain (21 kDa) as standards in 0·1 M sodium acetate buffer (pH 6·5). Additionally, SDS-PAGE electrophoresis was performed as described above to determine molecular weights and purity of the proteins.

RESULTS AND DISCUSSION

Isolation and purification of components of the *S. kurssanovii* chitinase complex were performed by a combination of three chromatographic steps, including metal–chelate affinity chromatography and hydrophobic-interaction chromatography on two gel beds with different hydrophobicity.

Four chitinases from the *S. kurssanovii* culture medium filtrate were purified to homogeneity. Figure 3 shows elution profiles at the final purification step of peak 6 by the hydrophobic-interaction chromatography. Two well-resolved peaks, 7 and 8, contained enzymes Chi-26 and Chi-42 completely separated from contaminating proteins, each showing a single band on disc gel electrophoresis (Fig. 4).

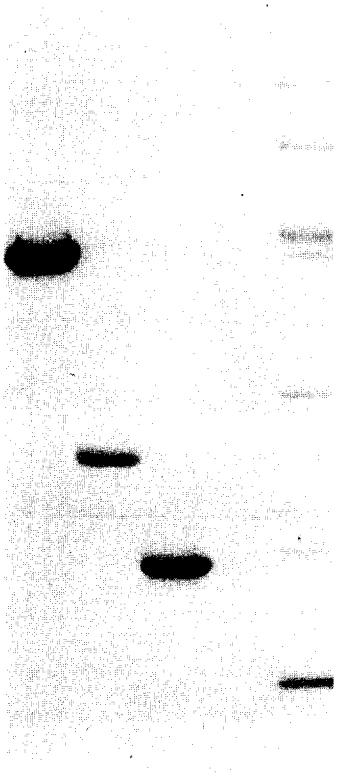

Fig. 4. Polyacrylamide gel electrophoresis of purified chitinases. SDS-PAGE electrophoresis was carried out with 10% gel using a discontinuous buffer system of Laemmli (1970). Standards of known molecular weight were run in the same gel and included the proteins of electrophoresis calibration kit of Pharmacia. The gel was stained for protein with Coomassie Blue G-250 (0·1% in 40% methanol, 10% acetic acid) and destained with 7·5% acetic acid. 1, Chi-42 (fraction 8); 2, Chi-26 (fraction 7); 3, Chi-20 (fraction 9); 4, markers.

By using the above combination of three chromatographic stages, the initial activity of Chi-42 was increased 34-fold, giving an overall activity yield of 30%. The final specific activity ranged from 8 to 10 U/mg of protein. Chi-26 was purified 22-fold, giving an overall yield of 13%, and its specific activity was typically 10 U/mg of protein. Two other chitinases (Chi-40 and Chi-20) were purified by chromatography on octyl-sepharose CL-4B (Figs 5 and 6, respectively) 2·2- and 4-fold only and represented 3 and 11% of the total activity, respectively.

It should be noted that the chitinase assay in the culture filtrate reflects the total enzymatic activity, whereby chitinases probably act synergistically on chitin (Tyunova *et al.*, 1973). In addition, a precise quantitative evaluation of the recovery and the purification factor is hardly possible because of our inability to distinguish between chitinase and chitobiase activities in the mixed preparation. Therefore, the activity evaluation of each single enzyme may appear lower than it is in reality.

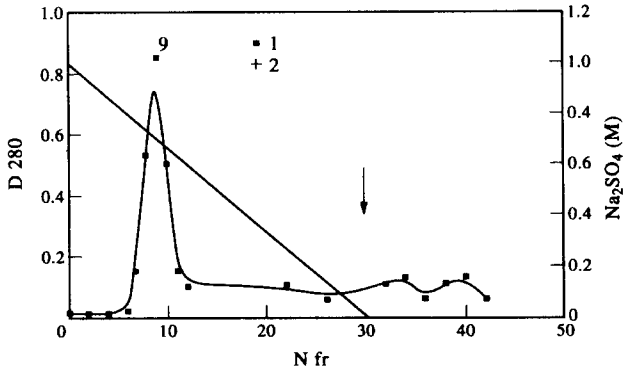

Fig. 5. Separation on octyl-sepharose CL-4B of fraction 5 (Fig. 2) obtained after chromatography on a butyl-toyopearl column. Conditions were the same as those given in the caption to Fig. 3. 1, Adsorption at 280 nm; 2, Na_2SO_4 concentration (M).

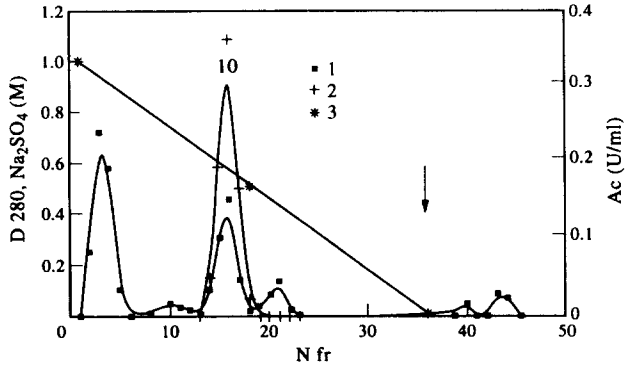

Fig. 6. Separation on octyl-sepharose CL-4B of fraction 2 obtained after chromatography on a Cu^{2+}–IDA–agarose column. Conditions were the same as those given in the caption to Fig. 3 except that the fraction volume was 3·2 ml. 1, Absorption at 280 nm; 2, chitinase activity (U/ml); 3, Na_2SO_4 concentration (M).

The final enzyme preparations were found to be homogeneous if judged by SDS-PAGE (Fig. 4). On the basis of migration distances, the four fractions corresponded to relative molecular weights of 42 kDa (peak 8), 40 kDa (peak 10), 26 kDa (peak 7) and 20 kDa (peak 9).

The relatively low recovery of Chi-40 and Chi-20 had permitted only limited characterization of these enzymes; 43% of the total chitinase activity was associated with two major components, Chi-42 and Chi-26, which were studied in more detail.

Native Chi-42 and Chi-26 were subjected to size-exclusion chromatography on Sephacryl S-200 using 0·1 M sodium phosphate buffer (pH 6·5) as the eluant; the resulting molecular weights were roughly estimated as 45 kDa and 30 kDa, respectively. These results imply the enzymes to be composed of single polypeptide chains. Both enzymes were subjected to analytical isoelectric focusing on polyacrylamide gels, with the result that the isoelectric points (pI values) corresponded to 7·1 (Chi-42) and 6·8 (Chi-26). The amino acid composition of each enzyme is given in Table 1.

We also examined basic biochemical characteristics of the purified enzymes. Chi-42 and Chi-26 showed broad pH stability in the pH region 3–9, with the pH optima of activity around pH 4·0 (Fig. 7a). This is consistent with the reported optima for other Streptomyces chitinases (Ueno *et al.*, 1990; Okazaki & Tagawa, 1991). Enzymes were stable at neutral pH for several weeks. Both chitinases showed temperature optima of activity at 50°C, but the activities fell sharply above this optimum temperature. Chi-26 had a higher thermal stability than Chi-42, which was found to have the highest stability at 25°C (Fig. 7b).

Table 1. Amino acid composition of chitinases (number of residues)

Amino acid	Chi-20	Chi-26	Chi-40	Chi-42
Asp	19·4 (19)	23·2 (23)	37·0 (37)	47·2 (47)
Thr	17·27 (17)	12·99 (13)	20·72 (21)	31·5 (32)
Ser	13·77 (14)	26·45 (27)	51·28 (51)	25·2 (25)
Glu	25·82 (26)	29·93 (30)	49·28 (49)	29·26 (29)
Pro	1·46 (2)	—	—	20·3 (20)
Gly	26·44 (26)	62·87 (63)	87·9 (88)	67·97 (68)
Ala	22·41 (22)	22·2 (22)	33·3 (33)	60·42 (60)
Cys	0·87 (1–2)	9·28 (10)	9·62 (10)	8·02 (8)
Val	8·42 (8)	14·27 (14)	24·53 (25)	20·39 (20)
Met	—	6·1 (6)	—	—
Ile	7·02 (7)	4·34 (4)	10·55 (11)	10·38 (10)
Leu	12·32 (12)	10·44 (10)	20·9 (21)	25·54 (26)
Tyr	6·5 (7)	8·89 (9)	8·81 (9)	22·66 (23)
Phe	11·58 (12)	8·21 (8)	10·1 (10)	18·88 (19)
Lys	8·98 (9)	5·82 (6)	11·84 (12)	24·78 (25)
His	4·33 (4)	3·41 (3)	5·88 (6)	4·81 (5)
Arg	10·3 (10)	3·5 (4)	6·6 (7)	7·22 (7)
Amount of residues	197	252	390	424

The effect of various divalent metal ions on the activity of the purified chitinases Chi-26 and Chi-42 was also tested (Table 2). We observed that Cu^{2+}, Hg^{2+}, Ni^{2+}, Sn^{2+} all behaved as strong inhibitors of enzyme activity at concentrations of 10^{-3} M. The ions Zn^{2+}, Mg^{2+} and Ca^{2+} had no effect on chitinase activity at the same concentration. None of the metal ions tested acted as an activator of the enzymes. EDTA at a concentration of 10^{-2} M had also no effect on the activity.

Rabbit antisera were prepared against purified chitinases Chi-42 and Chi-26. Antiserum prepared with Chi-42 cross-reacted with both Chi-42 and Chi-26, as did Chi-26 antiserum (Fig. 8). It is possible to conclude that at least two chitinases found in the culture filtrate of *S. kurssanovii* are immunologically related components which originated from a common ancestor.

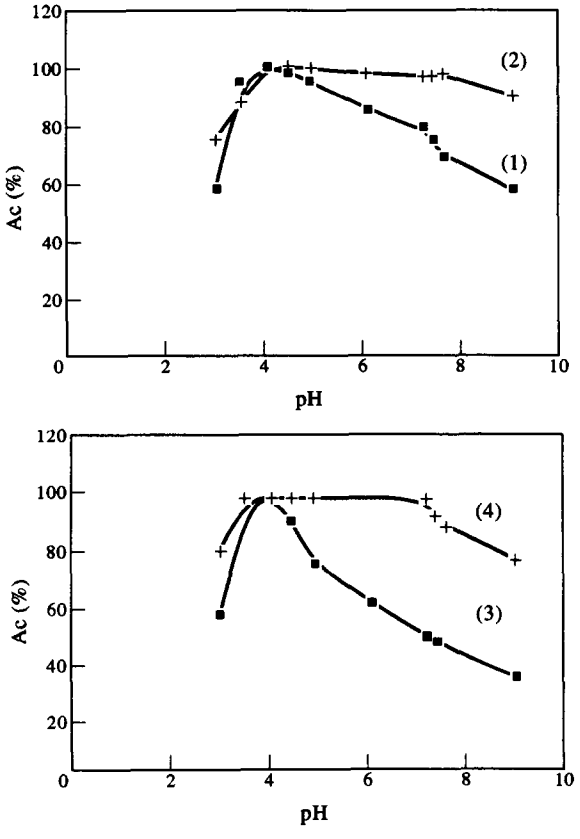

Fig. 7a. Effect of pH on activity (1) and stability (2) of Chi-26 (top) and on activity (3) and stability (4) of Chi-42 (bottom). Relative chitinase activities were determined as described in the text.

Table 2. Effect of metal ions on the activities of chitinases

Metal ion	Inhibition (%) of Chi-42	Inhibition (%) of Chi-26
Cu^{2+}	60	95
Sn^{2+}	50	63
Hg^{2+}	98	93
Ni^{2+}	35	68

However the origin and nature of multiplicity of *S. kurssanovii* chitinases are not clear.

The action of chitinases on chitooligosaccharides was investigated. Chitooligosaccharides with a degree of polymerization of $n = 3$–5 were incubated with Chi-42 and Chi-26 at 37°C for 5 and 30 min, and the reducing power of the resulting mixtures were determined. Both chitinases hydrolysed *N*-acetylchitotetraose and *N*-acetylchitopentaose at almost equal rates, faster than they hydrolysed *N*-acetylchitotriose. The products of hydrolysis were analysed by HPLC (Tables 3A and 3B). *N*-Acetylchitotriose was hydrolysed to *N*-acetylglucosamine and *N*-acetylchitobiose by both enzymes in 5 and 30 min. Both chitinases could also hydrolyse chitobiose, behaving like a chitobiase. *N*-Acetylchitotetraose was hydrolysed in 5 min to chitobiose, chitotriose and *N*-acetylglucosamine, and the pentamer was converted into chitotriose, chitobiose and GlcNAc as the main products. These results appear to indicate that Chi-42 and Chi-26 first hydrolysed *N*-acetylchitooligosaccharides to yield *N*-acetylchitobiose, *N*-acetylchitotriose and GlcNAc. When the reaction was allowed to proceed for 30 min, reaction products were partially converted back to higher chitooligosaccharides, especially by the action of Chi-26. In this case, the yields of higher *N*-acetylchitooligosaccharides were approximately 11% and 6% when the pentamer and tetramer were used as the initial substrates, respectively. From these results we proposed that Chi-26 acted on di-, tri- and tetramers of *N*-acetylglucosamine to produce *N*-acetylchitohexaose via chitinase-catalysed transglycosylation.

The chitinases Chi-42 and Chi-26 acted on colloidal chitin in an endo-splitting manner. Both enzymes also hydrolysed CM-chitin (69% substituted) and chitosan (85% deacetylated). The hydrolysis of soluble high-molecular-weight substrates was monitored by measuring the viscosity of the substrate solution, which is related to the chain length of the substrate. The action of Chi-42 and Chi-26 on chitosan and CM-chitin resulted

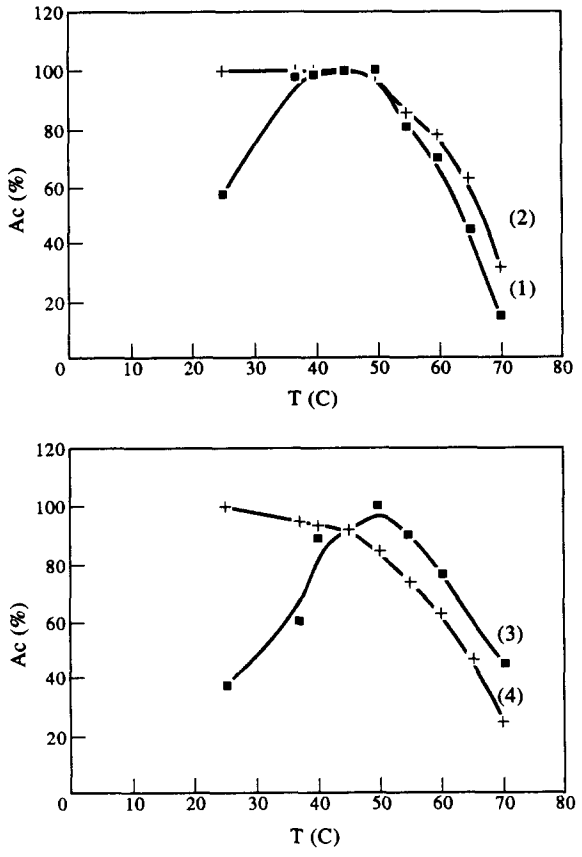

Fig. 7b. Effect of temperature on activity (1) and stability (2) of Chi-26 (top) and on activity (3) and stability (4) of Chi-42 (bottom).

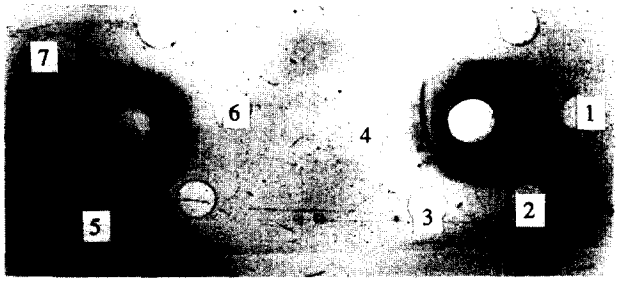

Fig. 8. Ouchterlony double immunodiffusion. Prior to diffusion the central well contained 50 µl of Chi-42 antiserum. Wells 1–4 contained Chi-26: 1, 0·05 mg; 2, 0·025 mg; 3, 0·0125 mg; 4, 0·01 mg. Wells 5–7 contained Chi-42: 5, 0·05 mg; 6, 0·025 mg; 7, 0·01 mg.

Table 3A. Enzymatic digestion products of *N*-acetylchitooligosaccharides obtained by incubation with Chi-42

N-Acetylchitooligosaccharide	Reaction time (min)	Digestion products[a] (%)					
		N-1	*N*-2	*N*-3	*N*-4	*N*-5	*N*-6
$(GlcNAc)_3$	5	62·9	7·0	29·8	—	—	—
$(GlcNAc)_3$	30	61·0	13·1	22·0	—	—	—
$(GlcNAc)_4$	5	59·5	13·4	27·6	0·5	—	—
$(GlcNAc)_4$	30	57·5	13·0	25·5	2·8	1·2	—
$(GlcNAc)_5$	5	54·5	8·5	23·0	3·2	6·2	—
$(GlcNAc)_5$	30	51·2	11·8	20·8	3·7	7·0	1·9

[a] Abbreviations: *N*-1 = *N*-acetylglucosamine; *N*-2 = *N*-acetylchitobiose; *N*-3 = *N*-acetylchitotriose; *N*-4 = *N*-acetylchitotetraose; *N*-5 = *N*-acetylchitopentaose; *N*-6 = *N*-acetylchitohexaose.

Table 3B. Enzymatic digestion products of *N*-acetylchitooligosaccharides obtained by incubation with Chi-26

N-Acetylchitooligosaccharide	Reaction time (min)	Digestion products[a] (%)					
		N-1	*N*-2	*N*-3	*N*-4	*N*-5	*N*-6
$(GlcNAc)_3$	5	76·3	10·5	13·2	—	—	—
$(GlcNAc)_3$	30	70·2	12·9	16·9	—	—	—
$(GlcNAc)_4$	5	63·8	12·5	23·7	—	—	—
$(GlcNAc)_4$	30	69·1	1·9	3·3	19·7	5·8	0·2
$(GlcNAc)_5$	5	65·7	12·8	18·5	2·0	0·5	—
$(GlcNAc)_5$	30	71·7	1·2	6·4	—	9·8	10·9

[a]Abbreviations: *N*-1 = *N*-acetylglucosamine; *N*-2 = *N*-acetylchitobiose; *N*-3 = *N*-acetylchitotriose; *N*-4 = *N*-acetylchitotetraose; *N*-5 = *N*-acetylchitopentaose; *N*-6 = *N*-acetylchitohexaose.

Table 4. Kinetic parameters of Chi-42

Substrate	V_{max} (μM/min)	K_m (μM)	V_{max}/K_m (min^{-1})
Chitosan	1·1	16	0·067
CM-chitin	0·5	18	0·028

in a rapid decrease in the viscosity of solutions of these polymers. Thus, Chi-42 and Chi-26 can be classified as 'randomly-acting' enzymes.

Kinetic studies were undertaken for chitinase Chi-42 only. As previously stated, Chi-42 hydrolysed chitosan and CM-chitin in an endo-splitting manner, so we found it appropriate to use the method of Rabinovich *et al.* (1977). The kinetics were evaluated by a viscosimetric method, which involved measuring the initial rates of decrease in viscosity of the polymer substrate, disregarding its nature and the nature of the enzyme. It is only important that the enzyme was an endo-depolymerase. The method allows us to evaluate V_{max} and K_m in the Michaelis–Menten kinetic scheme. The results are shown in Table 4. Although the K_m values of Chi-42 with both substrates are almost equal, the V_{max} values differ by a factor of two. So the catalytic efficiency of Chi-42 when measured by the relation V_{max}/K_m was twice as high for chitosan as it was for CM-chitin.

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