Kinetics of Immobilized Chitinase Produced by *Pseudomonas aeruginosa* K-187 in Shrimp and Crab Shell Fermentation

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Abstract

For immobilization, chitinase produced by *Pseudomonas aeruginosa* K-187 in shrimp and crab shell (SCS) fermentation was covalently bound to a pH-dependent hydroxypropyl methyl cellulose acetate succinate (ASL) polymer. It found that the immobilized chitinase was completely soluble at pH>5.5, while it became insoluble at pH<4.5. For this study, the immobilized efficiency as high as 99% was obtained when crude chitinase solution was used. The optimum pH and temperature increased from 6.0 and 40°C up to 8.0 and 50°C, respectively. The half-life for immobilized chitinase activity at 4°C was extended to 13 days. The ASL polymer is a water soluble and insoluble materials. It should be a better carrier for chitinase immobilization and activity retention than the water insoluble ones.

Keywords: Shrimp and crab shell waste, Chitinase, Chitin, Immobilized enzyme, Soluble-insoluble polymer, Hydroxymethylcellulose acetate succinate, *Pseudomonas aeruginosa*

Materials and methods

Hydroxypropyl methycellulose acetate succinate (ASL), molecular weight 93,000, was manufactured by Shin-Etsu Chemical Company, Japan. Ethylene glycol chitin (EGC), glycol chitin (GC), and 1-ethyl-3 (3-dimethylaminopropyl)-carbondiimide hydrochloride (EDC), were products of Sigma, USA. Trichloroacetic acid (TCA), carboxymethyl cellulose (CMC), casein, chitin powder, were reagent grade, Wako Chemicals, Japan. Shrimp and crab shell powder (SCSP), was purchased locally. Colloidal chitin was prepared from powdered chitin by the method of Jeniaux [1].

Crude chitinase solution

P. aeruginosa K-187 was cultured in a medium containing 3% shrimp and crab shell powder, 0.1% CMC, 0.1% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% $ZnSO_4$, 0.05% $MgSO_4$ $7H_2O$, under a pH of 7 and a temperature of $37°C$ for 2 days. After centrifugation, the culture supernatant was concentrated with ammonium sulfate precipitation. The resultant was precipitate was collected by centrifugation and dissolved in a phosphate buffer (pH 7), followed by dialysis against the same buffer.

Chitinase immobilization

1 g of ASL was dissolved in 20 ml of 0.1 M phosphate buffer (pH 7). This was mixed with a EDC solution made with 150 mg EDC in 5 ml water. The mixture was agitated for 20 minutes, before its pH was lowered to 4.5 with a phosphate buffer to precipitate ASL [2]. This procedure was repeated 3 times, and was added with 3 ml of crude chitinase solution. The reaction was allowed to last for 4 hours before the ASL precipitate was collected with phosphoric acid. The precipitate was washed with a citrate buffer (pH 4.5) three times.

Protein assay

Protein content was estimated by the method of Bradford [3], using Bio-Rad protein dye reagent concentrate. Bovine serum albumin was used as the standard.

Measurement of enzyme activity

Chitinase activity was measured with colloidal chitin as a substrate [4]. The amount of reducing sugar produced was measured by the method of Imoto and Yagishita [5] with N-acetylglucosamine as a reference compound.

For measuring protease activity, an appropriately diluted enzyme solution (0.2ml) was mixed with 2.5 ml of 1% casein in phosphate buffer (pH 7) and incubate for 10 min at $37°C$. The reaction was stopped by adding 5 ml of 0.19 M trichloroacetic acid. After centrifugation, the amount of soluble peptide in the supernatant was measured by the method of Todd [6] with tyrosine as a reference compound. One unit of protease activity was defined as the amount of enzyme which released 1 μmol of soluble peptides per hour.

The enzyme activities on GC, EGC, and CMC were assayed by the procedures as described previously [4, 7].

Results and discussion

Some animal and higher plant chitinase have lysozyme activity (chitinase/lysozyme), while bifunctional chitinase have not been isolated from microorganisms. Two chitinases produced by *P. aeruginosa* K-187 had antibacterial and cell lysis activities with many kinds of bacteria. This is the first report of a bifunctional chitinase/lysozyme from a microbe [4]. *P. aeruginosa* K-187, which was isolated from the soil of Northern Taiwan, was a producer of chitinase when cultured in SCSP [8]. Strain K-187 also has potential for producing chitinase using the acid and/or alkaline liquid waste from SCSP treatment. The results showed that strain K-187 can be applied to acid and/or alkaline liquid waste in the chitin production process [7].

Recovery of an enzyme after its application is a major concern in process economics. Immobilization is considered favorable in saving the enzyme for reuse. Insoluble immobilization undergoes heterogeneous reaction and its efficiency is limited by mass transfer. An alternative to this disadvantage is the use of a reversible soluble-insoluble carrier. With this carrier, homogeneous reactions can take place while afterward, the immobilizing complex is dissolved to recover the enzyme. The reversible carrier used in this study was supplied by Shin-Etsu Company. The original use of this carrier was for antacid capsulation. It changes solubility with the change of pH. Furthermore, it contains active functional group such as hydroxyl and carboxyl. These groups are suitable for binding enzymes.

When a crude chitinase of 7.4 mg/ml, activity of 2.6 U/l was the immobilizing enzyme, the immobilization conditions were optimal with 150 mg EDC, 3 ml enzyme, and 4 hours of immobilization time. Under these conditions, 99% of protein was immobilized. This percentage of immobilization was high compared to most other immobilization technique.

The most stable immobilization occurred at pH 7 between 6.0 and 8.0. Under this condition, the suspension was homogeneous, and the resulting enzyme was stable and was high in activity.

Hydrolysis of substrate chitin using free and immobilized chitinase is tested as a function of temperature. The optimum temperature for free and immobilized chitinase hydrolysis of chitin was 40 and 50°C, respectively. The free and immobilized chitinase remained stable for at least 30 minutes under these temperatures.

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From previous study, the enzyme produced by strain K-187 possessed the activity of chitinase and lysozyme [4]. Further to these properties, protease and cellulase activities were also discovered in this study. The enzyme had a higher activity to the water soluble GC than to CC, indicating hydrolysis was favored in homogeneous solution. For protease reaction with casein, a 20% activity remained after immobilization, indicating that these immobilization conditions were suitable for chitinase but not for protease.

The cold storage (4°C) free and immobilized enzymes were tested for their activity at different time span. A relative 100% was designated for enzyme activity at day 0. The times required for a 50% reduction of activity were 9 and 13 days, for free and immobilized enzyme, respectively.

The chitinase produced by strain K-187 was found antimicrobial. A test of 15 target strain, the immobilized chitinase was fatal to all strains except to strain K-187 itself. The effect was similar to free chitinase.

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COMPARISON OF ENDOCHITINASE ACTIVITIES AND ANTIFUNGAL PROPERTIES FROM NINE *SERRATIA* SPECIES

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Abstract

Nine strains of *Serratia* from various origins (ATCC, LMG, DSM) were cultivated on a medium containing acid casein digest, pure chitin or shrimp waste powder and mineral salts. Important variations were observed in protein contents and endochitinase activities. The protein contents ranged from 208 to 39 µg protein/ml; the chitinase activities from 45 to 1 U/ml. Seven strains inhibited the growth of *Fusarium oxysporum* at 50 µg prot/ml and two strains at 30 µg/ml without any relation to chitinase activity. For six *Serratia marcescens* strains, the use of shrimp waste powder resulted in higher protein content and chitinase activity. Chitinolytic enzymes were detected in SDS-PAGE gels using glycol chitin, 4-MU-GlcNAc and 4-MU-$(GlcNAc)_2$ as substrates. The fermented broths were demonstrated as fungitoxic and not fungistatic. Ammonium sulphate precipitation resulted in a loss of antifungal activity.

Keywords: chitin, chitinase, antifungal properties, *Serratia*, *Fusarium*.

Chitin, an insoluble linear ß-1,4- linked polymer of N-acetylglucosamine (GlcNAc) is widely distributed in nature as a principal structural component of the cell wall of fungi and of the exoskeletons of crustaceans and arthropods. Chitinase (EC 3.2.1.14) is an important chitin degrading enzyme which is involved in bioconversion processes of wastes from crustaceans and in plant protection by preserving them from chitin containing pathogens such as fungi.
Chitinases can be divided into two principal types: endochitinases and exochitinases. The final products formed by endochitinases are soluble low-molecular mass multimers of N-acetylglucosamine such as chitotetraose,

chitotriose and the dimer di-acetylchitobiose which is predominant. The product formed by exochitinase is di-acetylchitobiose which is hydrolysed into GlcNAc monomers in combination with ß- 1,4 N-acetylglucosaminidase (EC 3.2.1.30). When growing in a medium containing chitin as nitrogen source, *Serratia* species and especially *Serratia marcescens* produced extracellular chitinases. These enzymes are of major biotechnological interest because of their potential as "natural" antifungal agents. By studying endo and exochitinase activities of a mutant of *Serratia marcescens* ATCC 990 growing on chitin or fungus mycelia, the same antifungal activity was found for samples with low or high exo/endochitinase ratios[1]. This study aimed to compare endochitinase activities and antifungal properties from various wild types of *Serratia* species growing in the same culture conditions with as nitrogen source, pure chitin powder or dry shrimp waste powder.

Materials and methods

Preparation of colloïdal chitin
Colloidal chitin was prepared by stirring 10 g of pure chitin (Acros organics) into 100 ml of 85% phosphoric acid in the cold for 48 H. The mixture was diluted with 3L water and the precipitate washed up to neutrality[2]. The water suspension of colloïdal chitin was brought to 200 ml with water and passed through a 100 mesh sieve.

Determination of endochitinase activity.
5.0 g of the colloïdal chitin preparation was diluted to 50 ml with acetate buffer 20 mM pH 5.4 and 4 ml of this suspension was transferred into centrifuge tubes and maintained at 4°C until use. 1 ml of the centrifuged fermented broth was transferred in the tube and the absorbance at 700 nm was read. The tube was agitated in a water bath at 37° C for 30 min and the absorbance at 700 nm was read again.
One unit endochitinase activity corresponded to a decrease of 1% of the initial absorbance.

Protein determination.
Assays for total protein concentration in the broth were carried out using Coomassie blue reagent   (Coomassie Protein assay Reagent, Pierce) with bovine serum albumin (BSA) as the standard protein.

Determination of the antifungal activity.
To 5 ml of the centrifuged broth, 0.1 g of dehydrated PDB (Difco) was added. The solution was filtered (0.22 µm Millex-GP) in a small sterile bottle and 20 µl of a diluted mycelium suspension of *Fusarium oxysporum* (ATCC

16322) was added. The same procedure was carried out with the same broth heated 5 min at 100° C. The bottles were agitated for 48-72 H at 25°C. The mycelium suspension was centrifuged, the residue washed with water, centrifuged again and the dry weight was determined. The weight difference represented the percentage of the fungal growth inhibition.

Detection of chitinase activity after SDS-PAGE
Synthesis of glycol chitin, electrophoresis and detection of chitinase activity were carried out according to Trudel's methods[3]. Equal amounts of protein (5 µg) were applied per lane. For identification of the strains, see table 1. Chitinase activity was also visualized with fluorescent substrates in SDS-PAGE gels after renaturation in HEPES-KOH buffer (pH 7.0)[4].

Results and discussion

All the experiments were done in 500 ml flasks with 50 ml of the medium containing 1% acid casein digest,30mg% $MgSO_4 7H_2O$, 10 mg% $(NH_4)_2SO_4$, 14 mg% KH_2PO_4 and two chitin sources: pure chitin powder ≤100 mesh (0.5%) and shrimp waste powder (1%)obtained by grinding scampi shells previously dessicated at 100°C. The flasks were agitated for three days at 125 r/min and then centrifugated at 10000 r/min for 20 min.
Table 1 shows the results obtained with nine *Serratia* species. The first two columns give the protein concentration of the broth and its endochitinase activity; the other ones the antifungal activities of the broths diluted to 50, 30 or 20 µg protein/ml. The numbers printed in italic correspond to the experiments carried out with shrimp waste powder, the other ones with pure chitin.
Among the strains of *Serratia marcescens*, a great variation can be observed in the crude protein contents of the media after fermentation. One strain was found to produce 210 µg protein/ml with 45 U/ml; another one only 39 µg and 1.0 U/ml respectively. Two strains were found to produce 90-98 µg prot./ml with ± 9 U/ml. Three strains produced 67-78 µg prot./ml with 2-6 U/ml. *Serratia liquefaciens* and S. *plymutica* were characterized by a weak chitinase activity (2U/ml) with respectively 47 and 65 µg prot./ml.
All the strains inhibited the growth of *Fusarium oxysporum* at 50 µg prot./ml. Two strains only (H and I) reached the same antifungal capacity with 30 µg protein/ml. For two thirds of the strains, the presence of shrimp waste powder instead of pure chitin resulted in a higher protein content (153 - 185%). This increased protein concentration corresponded to a higher chitinase activi-

ty for the strains of *Serratia marcescens* but not for
S. liquefaciens and *S. plymutica*.
No improvement in antifungal capacity was observed by
using shrimp waste powder.

TABLE 1. Comparison of endochitinase activities (U/ml)
and antifungal properties (% *Fusarium* growth
inhibition) from nine *Serratia* strains.
The italic numbers correspond to experiments
carried out with shrimp waste powder, the other
ones with pure chitin.

Serratia strains				% inhibition		
		µg Prot /ml	U/ml	50 µg Prot/ml	30 µg Prot/ml	20 µg Prot/ml
S. marcescens						
ATCC 990	(D)	208	45	90	15	0
		197	*35*	*91*	*64*	*0*
DSM 1636	(L)	98	8	81	0	0
		150	*17*	*97*	*0*	*0*
LMG 2792	(E)	90	9	100	0	0
		101	*6*	*100*	*0*	*0*
LMG 3271	(N)	78	6	93	0	0
		144	*22*	*100*	*0*	*0*
ATCC 8100	(K)	70	2	94	0	0
		74	*2*	*100*	*0*	*0*
LMG 3276	(M)	67	4	98	0	0
		106	*19*	*100*	*0*	*0*
DSM 30121	(H)	39	1		100	96
		83	*7*	*100*	*69*	*37*
S. liquefaciens						
DSM 30065	(I)	47	2	100	98	0
		74	*2*	*100*	*100*	*0*
S. plymutica						
DSM 8571	(O)	65	3	96	9	0
		115	*2*	*97*	*0*	*0*

Characterization of the antifungal activity

- Are the broths fungistatic or fungitoxic?
Samples from *S. marcescens* (D) and (H), showing 100% inhibition after one week at 25°C were diluted with sterile PDB 2% to a protein concentration below 10 µg/ml and agitated for 6 days at 25°C. No mycelium development has been observed. This fact let us suggest a fungitoxic activity rather than a fungistatic one.

- Antifungal properties against Aspergillus and Botrytis
The results showed a good inhibition capacity for *Aspergillus niger* IHEM 2311. No inhibition was observed with *Botrytis cinerea* IHEM 3430.

- Influence of the protein precipitation by $(NH_4)_2SO_4$
Total proteins of the medium from the strain *S. marcescens* (D) were fractionated by ammonium sulphate. After desalting, the 35-90% fraction showed a 10-20% decrease of endochitinase activity, but did not inhibit fungus growth anymore, even at a concentration of 200 µg protein per ml. The loss of the antifungal activity in ammonium sulphate precipitated fractions was confirmed with other strains too.

Chitinase activities in SDS-PAGE

Several chitinase activities were detected in glycol chitin containing SDS-PAGE gels. *S. liquefaciens* (I) and *S. plymutica* (O) showed no bands when grown on chitin, but faint bands appeared when grown on shrimp waste. Strain H, which showed a weak endochitinase activity, showed the same chitinase banding pattern as strain D. Only strains D, L, E and N showed one clear chitinase band using 4-MU-$(GlcNAc)_2$ as substrate when grown on chitin. When grown on shrimp waste D, N and M showed one band with this substrate, while L and E showed no activity any more.
Using 4-MU-GlcNAc as substrate, only strain D showed a strong activity, while strain H showed one weak band on chitin as well as on shrimp waste.

Conclusion

Changes in protein contents and endochitinase activities were observed in various *Serratia* species and especially in *Serratia marcescens* strains growing on medium containing chitin. For most of them, the use of shrimp waste powder instead of chitin resulted in higher protein contents and chitinase activities. There is no correlation between banding patterns, spectrophotometrically determined endochitinase activities and antifungal properties. Protein precipitation by ammonium sulphate resulted in a loss of the fungitoxic capacity.

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INFLUENCE OF CULTIVATION CONDITIONS
ON THE ACTIVITY OF CHITIN DEACETYLASE FROM *MUCOR ROUXII*

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Abstract

Chitin deacetylase is studied to elucidate its biological role and the possibility of using the enzyme in the deacetylation of chitinous material. *M. rouxii* may be a convenient source of deacetylase, because the biomass, after the enzyme isolation may be used for isolating chitosan. The cultivation conditions should be suitable for assuring enzyme production and efficient growth of *M. rouxii*. The effect of pH, time of incubation, and composition of the culture media on deacetylase production in *M rouxii* was studied. The specific activity of deacetylase in the crude extract from *M. rouxii* cultivated in a liquid YPG medium in a shaker-bath was maximal after 12-96 h, and the total activity after 48-96 h. The total activity of extracellular deacetylase had a slight minimum at about 48 h followed by a maximum after 96 h of incubation. Cultivation in YPG medium at pH 5 was optimal, considering both specific and total activity of the intracellular deacetylase. The specific activity in the extracts from *M. rouxii* cultivated in YPG and in a defined medium (glucose, glutamate, mineral salts, vitamins) was similar, while the total activity was two times higher when YPG medium was used. The specific and total activity in the extracts from mycelium of *M. rouxii* cultivated in YPG medium containing digitonin (0.1 mg/1 ml) was higher when compared to that of the control culture. During 12 - 62 h the effect of digitonin increased with the time of incubation. Replacing peptone in the YPG or glutamate in the defined medium by sterile biomass of the fungi increased the production of the intracellular deacetylase by *M. rouxii* and decreased the total activity of deacetylase in the culture filtrate.

Keywords: Mucor rouxii, chitin deacetylase, factors affecting activity, chitosan

Materials and methods

Materials

Alumina A-5 and digitonin were from Sigma; kit for determination of acetic acid was from Boehringer. Other chemicals used were also of analytical grade. Chitosan deacetylated in 61% (chitosan-61) was prepared from Antarctic krill shells in Sea Fisheries Institute in Gdynia.

Effect of cultivation conditions on growth of M. rouxii and chitin deacetylase activity

About 10^7 spores of *M. rouxii* ATCC 24905 were inoculated per 100 ml of appropriate liquid medium. The cultures were incubated in a reciprocating shaker bath at 28°C.

To determine the effect of incubation time on the deacetylase activity *M. rouxii* was grown in liquid YPG medium (0.3% Difco yeast extract, 1% Difco peptone, 2% glucose)

at initial pH 4.5, while for the determination of the effect of pH the pH was adjusted in the range 3 - 7.

The effect of composition of the culture medium was investigated using:
- YPG containing 0.1 mg digitonin in 1 ml of the medium
- YPG, in which peptone was replaced by freeze-dried and sterilised *M. rouxii* mycelium
- defined medium: glucose - 20g, sodium glutamate - 6.7 g, thiamine - 1.0 mg, nicotinic acid - 1.0 mg, $KH_2PO_4 \times 7\ H_2O$ - 1.0 mg, $MnSO_4 \times H_2O$ - 0.3 mg, $CuSO_4 \times 5\ H_2O$ - 0.4 mg per 1000 ml of the medium
- defined medium, in which glutamate was replaced with freeze-dried and sterilised *M. rouxii* mycelium.

In all experiments, carried out in triplicate, the mycelium was separated from the culture medium by filtration on a Büchner funnel with filter paper or through a double layer of gauze and filter paper, washed with cold, distilled water, and frozen at -20°C. The fraction retained on the gauze contained long filaments, and that on the filter paper - arthrospores and shorter filaments.

The standard deviation of the results was within 5 - 11% of the respective mean values.

Preparation of the mycelial extract

The frozen mycelium was ground 15 min with Alumina A-5 (1:2, w/w) in a mortar, at 0°C. During grinding, 0.025 M Tris-HCl buffer pH 7.2, was gradually added (wet mycelium: buffer = 1:7, w/v). The homogenate was centrifuged at 0°C for 10 min at 4000 × g, and for 60 min at 15000 × g. The protein concentration in the extract was determined according to Lowry et al.[1] with bovine serum albumin as a standard.

Enzyme assay

The activity of chitin deacetylase was assayed by determining acetic acid released from the substrate. The enzymatic method of Bergmeyer and Möllering[2] was used.

The reaction mixture, containing 2.5 mg of the chitosan-61 and 25 - 50 μl of the extracts or culture filtrate in 1 ml of 0.1 M Tris-HCl was incubated at pH 5.8 for 30 min at 40°C. The reaction was terminated by heating the samples for 3 min in a boiling water-bath. The pH in each sample was adjusted to 8-9 by NaOH addition in order to avoid chitosan precipitation during the determination of acetic acid. The samples were centrifuged for 20 min at 15000 × g and acetic acid was determined in the supernatant. The specific activity of the enzyme was expressed as mU mg^{-1} protein. One unit is defined as the amount of enzyme that produces 1 μmole of acetic acid of chitosan-61 in 1 min under the conditions of the assay.

Results and discussion

Effect of time of incubation on the growth of M. rouxii and on the activity of chitin deacetylase

Maximum growth rate of *M. rouxii* was observed during first 48 h of incubation at 28°C (Fig. 1.). After 96 h the quantity of the biomass reached a plateau. Bartnicki-Garcia and Nickerson[3] obtained similar biomass yields after 48 h of incubation, in aerated *M. rouxii* cultures.

The quantity of intracellular protein produced by *M. rouxii* reached a maximum after 48 h of incubation. It decreased significantly after 72 h, in spite of the fact that the biomass yield was stable (Fig. 1.), what suggests that excretion of proteins out of the cells was not the reason of this decrease.
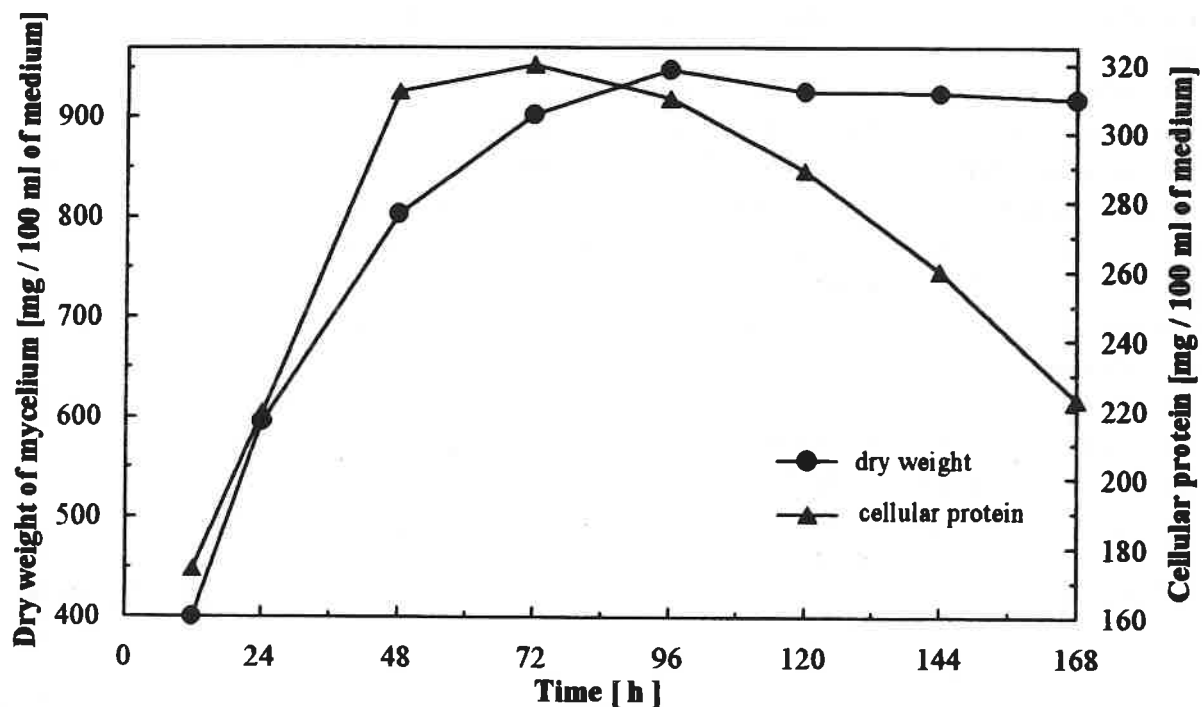
Fig. 1. Effect of incubation time on *M. rouxii* growth in YPG medium
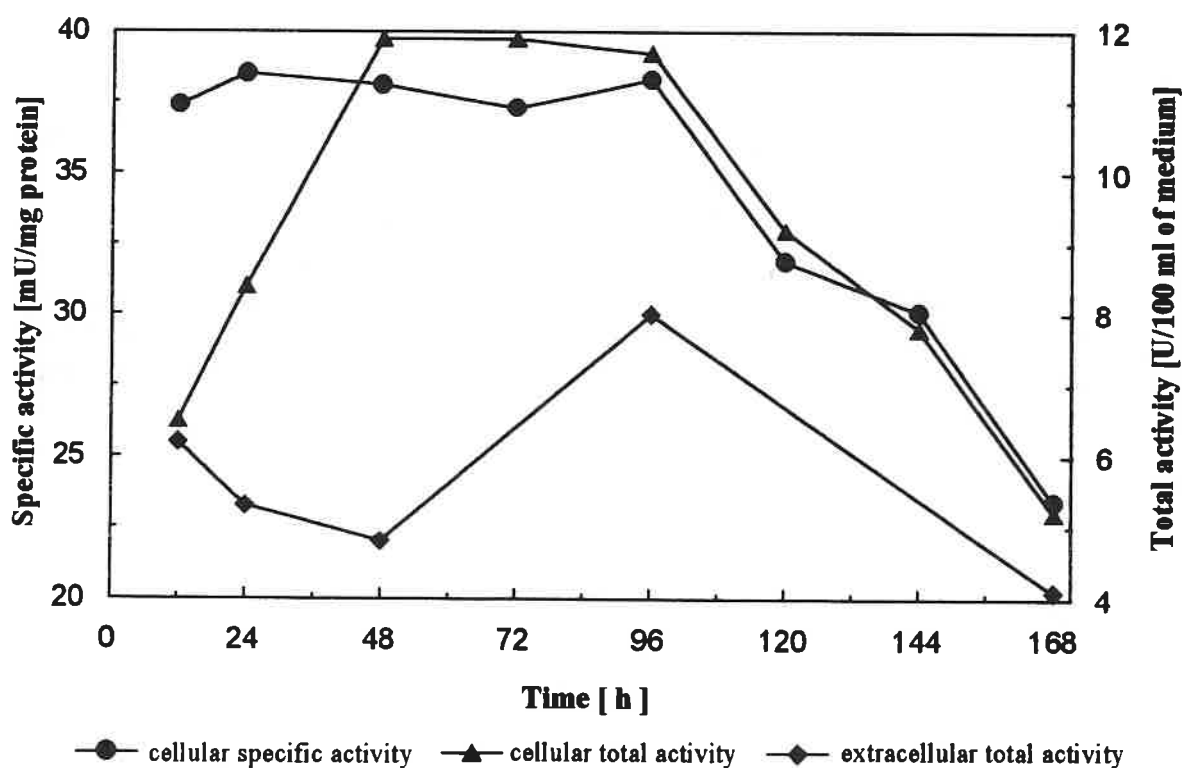
Fig. 2. Effect of incubation time of *M. rouxii* in YPG medium on chitin deacetylase activity

The specific deacetylase activity in the extract of the mycelium from 12-96 h cultures remained constant (Fig. 2.). In the extracts obtained from older cultures the specific activity decreased, just as the protein yield. Dunkel and Knorr[4] found that the specific deacetylase activity reached a maximum in the extracts obtained from aerated *M. rouxii* cultures

incubated for 15 h. The total deacetylase activity in the extracts corresponded with the protein content, reaching a maximum in the extracts from 48 h cultures (Fig. 2.).

Chitin deacetylase is also excreted extracellularly[5]. Therefore also the enzyme activity in the culture filtrate was determined. It was found that up to 48 h of incubation, the total deacetylase activity in the culture filtrate slightly decreased and reached a maximum in 4 days' cultures (Fig. 2.). As for now, it is difficult to explain this enhanced activity, especially that intracellular protein concentration and deacetylase activity remained stable. Further experiments are necessary to confirm the results and to determine the quantity of the protein excreted by the cells during incubation.

Tab. 1. Deaceylase activity, depending on the morphological form of *M. rouxii*[a]

Morphological form	Dry weight [mg/100ml of medium]	Protein [mg/100 ml of medium]	Specific activity [mU/mg of protein]	Total activity [U]
Long hyphae	802	207	41.9	8.7
Short fragments of hyphae and arthrospores	303	79	32.1	2.5

[a] YPG medium, 96 h cultivation

Bartnicki-Garcia and Nickerson[6] proved that the quantity of arthrospores and short hyphae increased with the time of incubation. That is why it seemed reasonable to investigate the effect of morphological form of the culture on the enzymes' activity. The fraction, containing short hyphae and arthrospores, obtained from 4 days' cultivation, constituted about 27% of total biomass and the same percentage of protein. The specific activity of the extract from long hyphae was about 30% higher than in the fraction containing arthrospores and short hyphae (Tab. 1).

Effect of pH on M. rouxii growth and on the activity of chitin deacetylase

M. rouxii growth did not depend on pH in the studied range (3.1 - 7.0), while the amount of protein slightly increased when pH exceeded 4. Specific and total deacetylase activity was minimal in the extracts obtained from *M. rouxii* cultivated at pH 3.1, while the activity of deacetylase present in the culture filtrate was maximal in the same conditions. In respect of the specific and total activity of intracellular deacetylase, the optimum pH was about 5 (Fig. 3.).

Effect of culture medium composition on M. rouxii growth and on the activity of chitin deacetylase

Bartnicki-Garcia[7] showed that the chitosomes isolated from *M. rouxii* cells dissociated, when treated with digitonin. The chitin synthetase from these dissociated chitosomes and the chitin deacetylase produced *in vitro* more chitosan from UDP-GlcNAc, than in experiments with non-dissociated subunits. It seemed interesting to check if digitonin in the culture medium enhances the production of chitin deacetylase by *M. rouxii*. It was found that digitonin addition to YPG medium did not significantly change the yield of biomass, or the intracellular protein content in the cultures incubated for 12 - 62 hours (Fig. 4.). On the other hand, digitonin in the medium increased the specific and total deacetylase activ-

ity in the extracts from *M. rouxii* cells. The difference in the activity increased with the incubation time. Specific deacetylase activity in the extracts from the cells, incubated for 62 h in the culture medium containing digitonin, was about 70% higher, in comparison with the control culture. It is possible that higher activity in the presence of digitonin was partially caused by releasing the enzyme from the particulate cell fractions. After 12 h of incubation, the total activity of the excreted deacetylase was lower in the cultures with digitonin addition. In the older cultures this tendency was not observed.
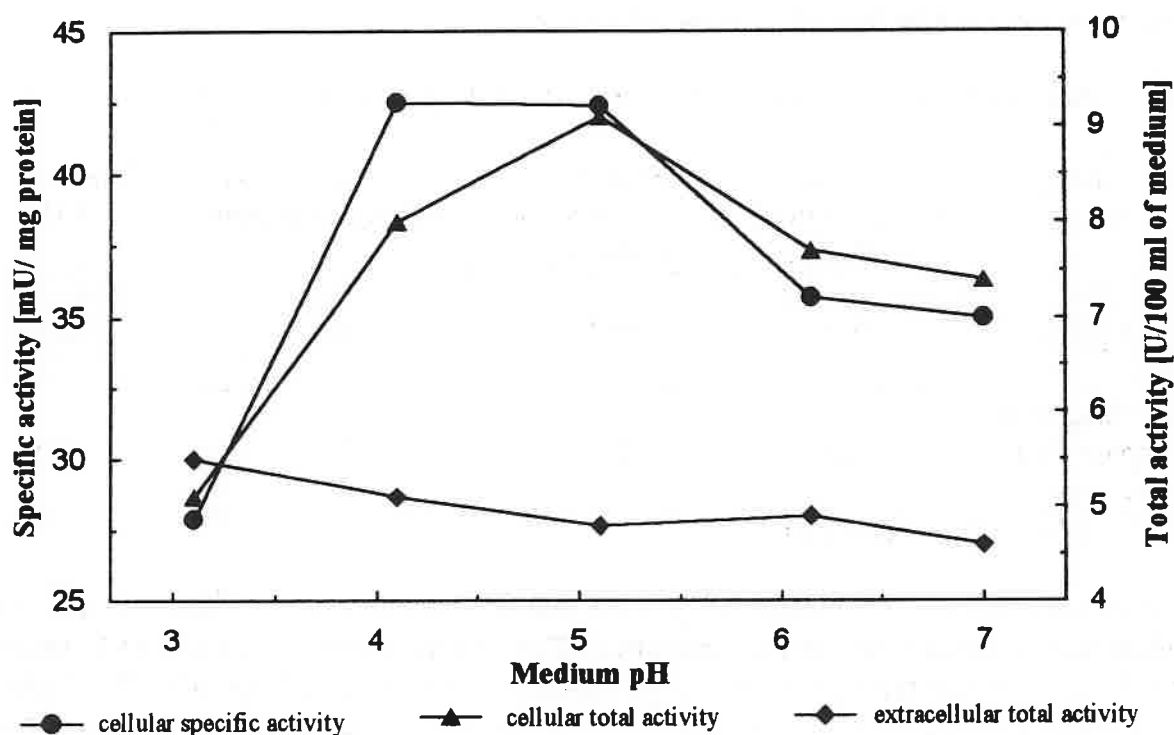

Fig. 3. Effect of *M. rouxii* cultivation, 24 h in YPG medium at different pH, on chitin deacetylase activity
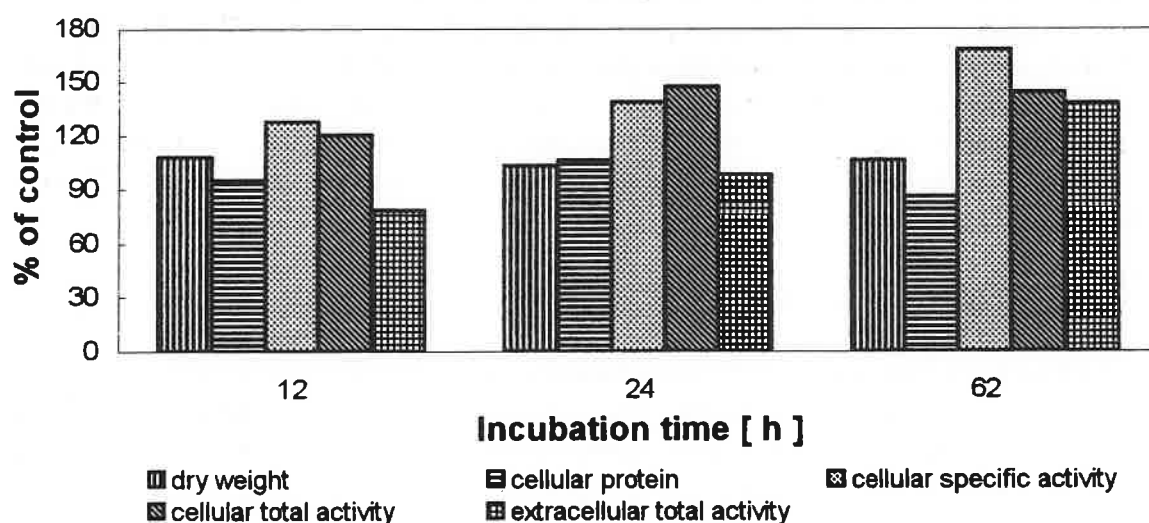

Fig. 4. Effect of *M. rouxii* cultivation in YPG medium with digitonin addition on chitin deacetylase activity. Control - *M. rouxii* cultivated in YPG medium without digitonin addition

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According to Dunkel and Knorr[4] addition of chitin of microbial origin to *M. rouxii* and *Phycomyces blakesleenus* cultures caused an increase of chitosan content in the cell walls of these fungi and water-soluble chitosan deacetylated in 41% enhanced deacetylase activity as well. *M. rouxii* biomass, in spite of containing rather small amounts of chitin and chitosan acetylated only in 3-27%[8,9] enhanced the intracellular deacetylase activity in 48 and 96 h cultures, when added to YPG or defined medium (Fig. 5). The difference in the activity increased with time. Sterilised fungi biomass in the media decreased the total activity of the deacetylase excreted out of the cells (Fig. 5).

a)   b)

☰ cellular protein   ▨ cellular specific activity   ▧ cellular total activity   ▤ extracellular total activity
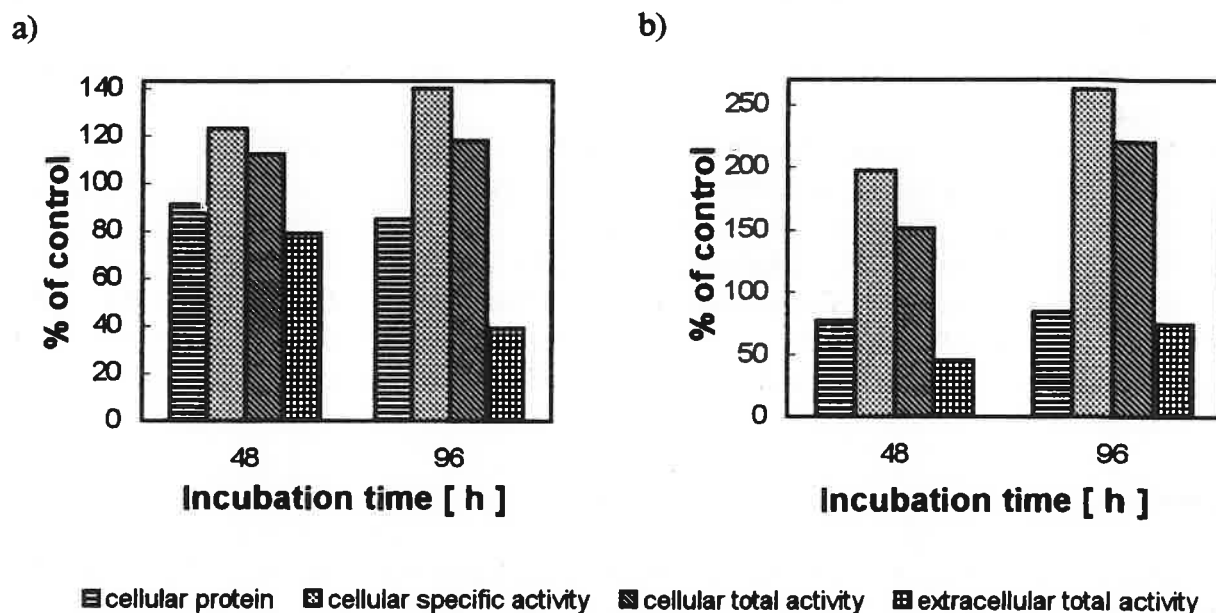
Fig. 5. Effect of replacement of peptone in YPG medium (a) and glutamate in defined medium by sterile biomass of *M. rouxii* on growth of fungi and chitin deacetylase activity.
Control - *M. rouxii* cultivated in non-modified medium

Tab 2. Deacetylase activity and growth of *M. rouxii* in YPG and defined medium

Medium	Incubation time [h]	Cellular protein [mg/100 ml of medium]	Specific activity [mU/mg of protein]	Total activity [U]
YPG		303	37.5	11.4
	48			
Defined medium		165	39.8	6.6
YPG		286	39.8	11.4
	96			
Defined medium		166	33.5	5.6

The specific deacetylase activity of the extracts from *M. rouxii* cells cultivated in YPG and defined media was similar, while the total activity in the case of YPG medium was twice as large as for the defined one. This was caused by better growth of *M. rouxii* in YPG medium, manifested by higher yield of protein (Tab. 2.).

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Conclusion

M. rouxii is a convenient source of deacetylase. It is possible to enhance the enzyme yield by modification of the cultivation conditions of these fungi. The biomass after enzyme isolation may be used as a component of the medium for *M. rouxii* growth. It simultaneously improves the enzyme production.

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Acknowledgement

EXPRESSION AND CHARACTERIZATION OF RECOMBINANT CHITIN DEACETYLASE

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Abstract

Chitin deacetylase (CDA) gene from *Mucor rouxii* was cloned into an integrative plasmid in the methylotrophic yeast *Pichia pastoris*. CDA gene expression resulted in efficient secretion of biologically active chitin deacetylase in the extracellular medium. A time-course study of recombinant CDA production was performed in 2 1-shake flasks and 30 1-fermentor. The properties of the enzyme were analyzed and compared with the characteristics of other native chitin deacetylases.

Keywords: Chitin, chitosan, chitin deacetylase, Mucor rouxii, yeast, Pichia pastoris.

Introduction

Chitosan, the deacetylated form of chitin, is generally found in the cell walls of Zygomycetes. This biopolymer has a broad varity of applications in the fields of agriculture, food-additives, cosmetics, health,... Commercial chitosan is traditionnally produced by thermochemical deacetylation of chitin from crustacean shells. However, the very drastic conditions of the chemical process generally induce great variability in deacetylation degree and chain degradations. An alternative procedure based on the enzymatic deacetylation of chitin could be an improved method for producing chitosan with well-controlled characteristics.

Chitin deacetylase, the enzyme that catalyzes the conversion of chitin to chitosan, was first identified in extracts of the fungus *Mucor rouxii* (1). It was reported that chitosan biosynthesis occurs by deacetylation of nascent chitin chains through the action of chitin deacetylase (2). Recently chitin deacetylase was purified to homogeneity from *M. rouxii* (3) and *Colletotrichum lindemuthianum* (4) and further characterized. A cDNA of *M. rouxii* encoding chitin deacetylase was isolated and sequenced (5).

In order to evaluate the potential use of an enzymatic way for chitosan production, we analyzed the feasibility to produce chitin deacetylase at a large scale. In this paper, we report the expression of *M. rouxii* chitin deacetylase in a recombinant microorganism, the methylotrophic yeast *Pichia pastoris*. The properties of the recombinant protein were analyzed and compared with the characteristics of other native chitin deacetylases.

Material and methods

Plasmid and strains. Plasmid and yeast strain were purchased from Invitrogen (Pichia Expression Kit). The host strain used for *Pichia pastoris* transformation was GS115, a histidine-requiring strain. The plasmid was pPIC9, an integrating plasmid containing the methanol inducible AOX1 promoter, the HIS4 selectable marker and the *S. cerevisiae* α-factor secretion signal sequence.

Recombinant DNA techniques. The techniques for cDNA cloning in the expression vector, transformation of *P. pastoris* and screening of transformants for chitin deacetylase expression were previously described (6). The strain used for chitin deacetylase expression and production experiments was the best producing clone selected among His⁺Mutˢ transformants.

Media and culture conditions. All cultures were grown in FM21 minimal medium supplemented with either glycerol or methanol as sole carbon source. For 2 1-flask studies, recombinant P. pastoris strain was grown at 30°C with shaking. Upon glycerol exhaustion (~ 24 hours), the cultures were induced by adding 0.5% methanol every day. Fermentor cultures were performed in a 30-liter New Brunswick Scientific fermentor. The cultures were run acoording to the Pichia fermentation process guidelines established by Invitrogen. After fermentation, the supernatant was collected, concentrated by ultrafiltration (Amicon, S10Y10 cartridge) and lyophilized.

Enzyme assays. Two different methods were used for determining chitin deacetylase activity.

i) Radiometric assay: chitin deacetylase activity was estimated by using as substrate a water-soluble chitin derivative (glycol chitin) radiolabelled in N-acetyl groups. The substrate was prepared according to Araki and Ito (1). The enzyme assays were performed in the same conditions as Kafetzopoulos et al (3).

ii) Enzymatic assay: Acetic acid released by the action of chitin deacetylase on chitininous substrate was determined by the enzymatic method of Bergmeyer (7).

Protein analysis. Electrophoresis in SDS-polyacrylamide gels were performed as described by Laemmli (8). Protein bands were visualized with Coomassie brilliant blue R or silver. For western blot analysis, proteins were transferred onto nitrocellulose membranes. Chitin deacetylase was visualized using a rabbit polyclonal antiserum raised against *M. rouxii* chitin deacetylase, and a second antibody conjugated to alkaline phosphatase. Protein content was determined by the method of Lowry et al (9) using bovine serum albumine as standard. Enzymatic deglycosylation assays were performed with Endoglycosidase H from *Streptomyces plicatus* under native and denaturing conditions. Molecular weight of the recombinant protein was determined by gel filtration on Superdex 75HR 10/30 (Pharmacia Biotech).

Results and discussion

Expression of chitin deacetylase.

The chitin deacetylase (CDA) expression was examined in 2 1-shake flasks and in 30 1-fermentor. Induction lasted to 120 hours and a time-course study of protein production was performed during this period (fig.1). The CDA expressed in *Pichia pastoris* was secreted in the culture medium and no enzyme activity was detected in cell lysates.
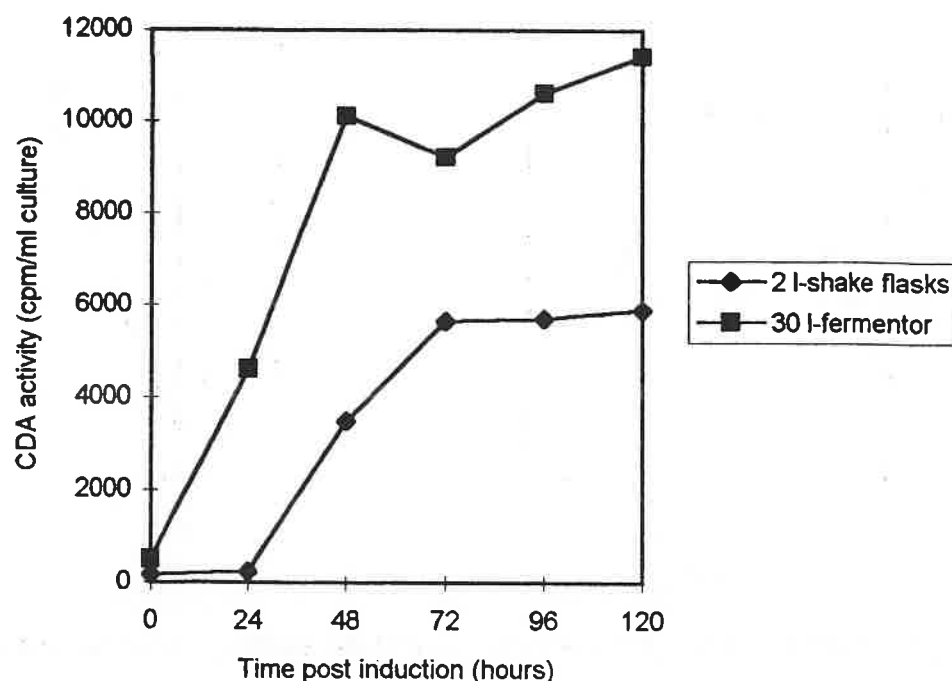
Fig. 1. Time-course of recombinant chitin deacetylase production.

In shake flasks, after a 24 h latent period, the expression of CDA increased rapidly and the maximum level was attained within 72 h induction ; thereafter the expression level remained fairly constant. In fermentor, the expression of CDA was directly induced upon switching to methanol medium and reached a maximum within 48 h induction. After this time, the expression level increased slightly and a second maximum of CDA production was obtained after 120 h induction. In the preliminar trials, the expression yield of CDA in fermentor was twice as that observed in shake flasks ; it corresponds to ~ 40 mg CDA/l culture.

Characterization of the recombinant chitin deacetylase

The apparent molecular mass of recombinant CDA was estimated to be ~75-80 kDa by electrophoresis on SDS-polyacrylamide gels and gel filtration on Superdex 75HR 10/30. The ~ 80 kDa recombinant protein was recognized by rabbit polyclonal antiserum raised against the native enzyme from M. rouxii. However, electrophoretical analysis showed also a heterogeneous product with a molecular weight > 100 kDa, appearing as a heterogeneous immunoreactive smear on western blots and suggesting that the recombinant secreted product was partly hyperglycosylated. SDS-PAGE analysis of recombinant enzyme after enzymatic deglycosylation by EndoH revealed a major protein band at ~ 60 kDa. Deglycosylation of CDA in native conditions resulted in a total loss of enzyme activity.

The results concerning the effects of pH and temperature on the stability and the activity of the recombinant protein are shown on fig. 2a-b and 3a-b. The recombinant CDA was stable in a narrow pH range from 4.0 to 5.0 and the optimum activity was detected at pH 5.0. The enzyme activity reached maximum at 60°C and the recombinant CDA exhibited a good thermostability: 100% of initial activity until 50°C.
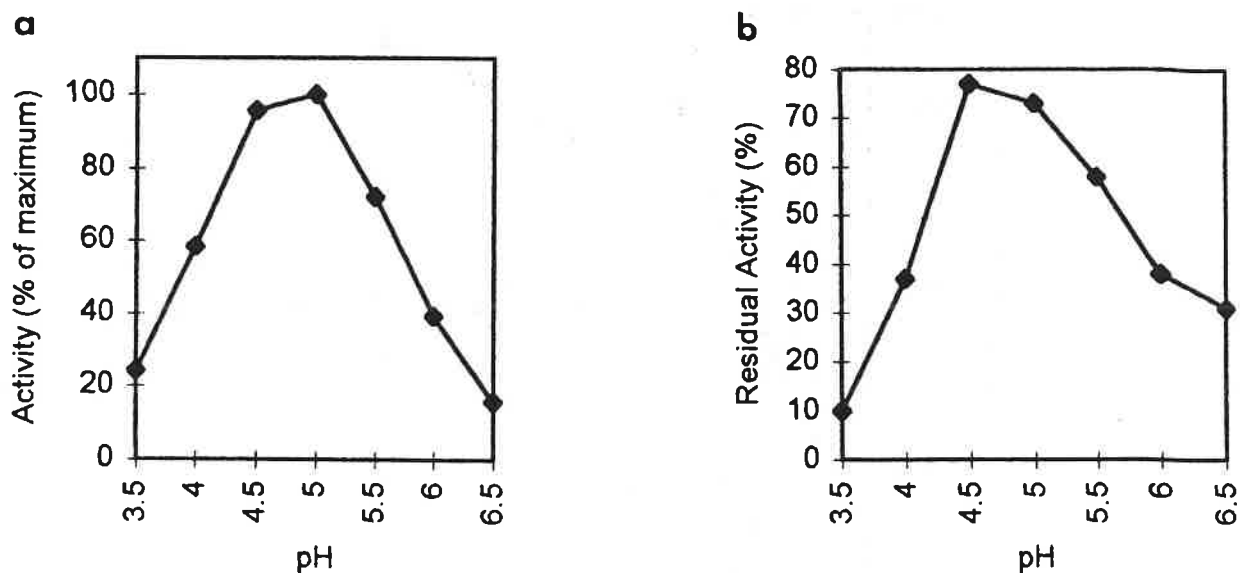
Fig. 2. Effect of pH on the activity (a) and the stability (b) of recombinant CDA.
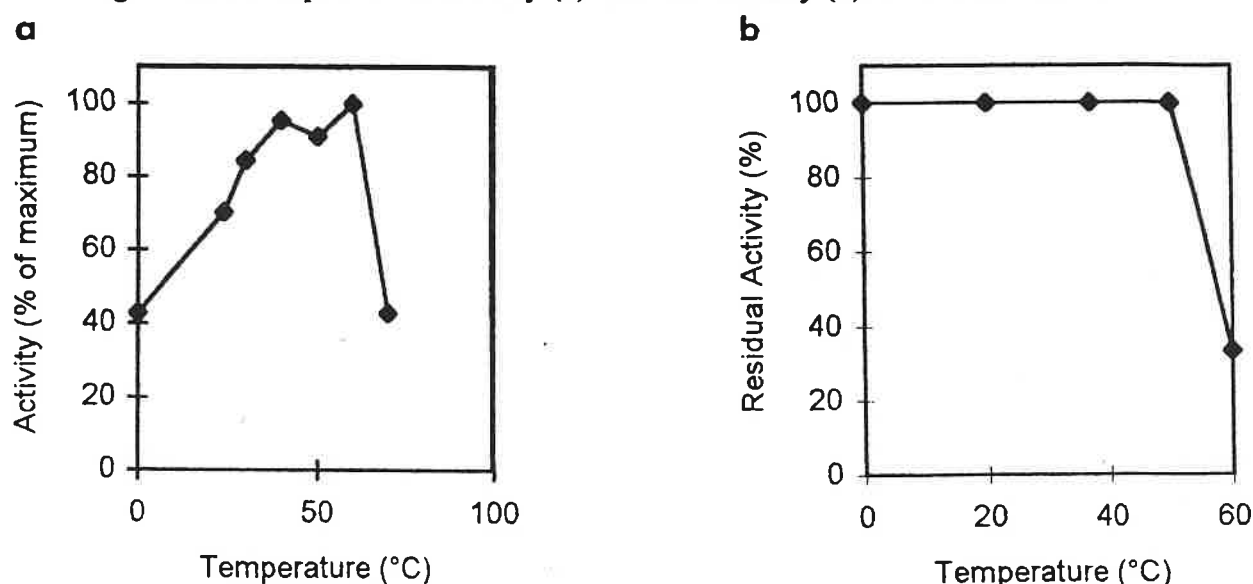
Fig. 3. Effect of temperature on the activity (a) and the stability (b) of CDA.

The specificity of the recombinant enzyme for various substrates was investigated and the results are shown in Table I. N-acetylglucosamine was poorly hydrolyzed by recombinant CDA. The enzyme exhibited activity in presence of chitin-hexamer, soluble CM-chitin and insoluble colloidal chitin. However in all cases the enzyme activity was lower than with glycolchitin. With partially N-deacetylated chitosan as substrate, the enzyme activity was as high as with glycolchitin.

The recombinant CDA did not require any metal ions for activity. Co^{++}, Mg^{++}, Mn^{++} and Zn^{++} only slightly influenced enzyme activity at very low concentrations (<10 mM) and they were inhibitory at concentrations exceeding 10 mM. Cu^{++} strongly inhibited CDA activity even at 1 mM. The influence of acetate on enzyme activity was also examined. The recombinant protein exhibited a good stability in presence of acetate: ~ 90% of initial activity in 10 mM acetate and ~ 65% in 200 mM acetate, after 6 hour's incubation.

Table I. Substrate specificity of recombinant chitin deacetylase.

Substrate	Relative activity (%)
Glycol chitin	100
GlcNAc	6
(GlcNAc)$_6$	75
CM-chitin	26
Colloidal chitin	16
Partially N-deacetylated Chitosan	100

The concentration of the substrates was adjusted in respect to their N-Acetyl residues content. The assays were performed during 24 hours at 37°C.

Conclusion

The methylotrophic yeast *Pichia pastoris* has been shown to be an efficient host system for the production of large quantities of foreign proteins (10). This yeast system was successfully used for the expression of chitin deacetylase gene from *Mucor rouxii*. The expressed protein was biologically active and secreted in the culture medium, which is advantageous for downstream product recovery.

Recombinant and native enzymes have several similar characteristics. For example, the two enzymes are glycosylated and they have the same apparent molecular weight (~ 75-80 kDa) and similar pH and temperature optimum. However chitin deacetylase expressed in *P. pastoris* exhibited a higher thermostability and a better stability in presence of acetate. Finally it was shown that the recombinant enzyme hydrolyzed efficiently chitinous substrates as chitohexaose, carboxymethylchitin, glycol chitin, insoluble colloidal chitin and partially N-deacetylated chitosan. Although the activity was relatively weak, *P. pastoris* chitin deacetylase was active on GlcNAc, which is quite different from natural chitin deacetylases from *M. rouxii* and *Colletotrichum lindemuthianum* (3, 4).

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A NEW APPROACH TO THE SYNTHESIS OF CHITINASE INHIBITORS RELATED TO POLYOXINS. APPLICATION TO A DIDEOXYPOLYOXIN C COMPOUND.

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Abstract. Optically active tert-butyl-(4,5-epoxy-3-hydroxy)-esters have been used to prepare the 2'-deoxy nucleosidic part of thymin polyoxin analogue **17**, in 8 steps and 15% overall yield. The main steps concern the stereo- and regioselective opening of the epoxide ring by an azido anion, the stereoselective introduction of the thymine base and the transformation of the primary alcohol to the acid functionality of the final product.

Materials and methods.

Commercially available reagents were used as supplied. All solvents were distilled before use. Products were purified by medium pressure liquid chromatography on a Jobin et Yvon Moduloprep apparatus by using Amicon 6-35 μm silica. NMR spectra were recorded with a Bruker AC-250 spectrometer. Mass spectra were recorded on a Nermag R10-10 apparatus. Elemental analyses were carried out by the "Service Commun de Microanalyse élémentaire UPS-INP" in Toulouse.

Results and discussion.

The enzyme chitin synthase (EC 2.4.1.16)[1] catalyses the production of chitin the $\beta 1 \rightarrow 4$-linked polymer of N-acetylglucosamine (GlcNAc) which is one of the most common polysaccharides and one of the major structural components of the cell wall of most fungi.[2]

Polyoxins (fig. 1) form an important class of peptidyl nucleosides isolated from the culture broths of *Streptomyces cacaoi var. asoensis*[3] and were found to be potent inhibitors of chitin synthase.[4] Polyoxin D is used as an agricultural antifungal agent to treat rice sheath blight and pear black spot.[5]
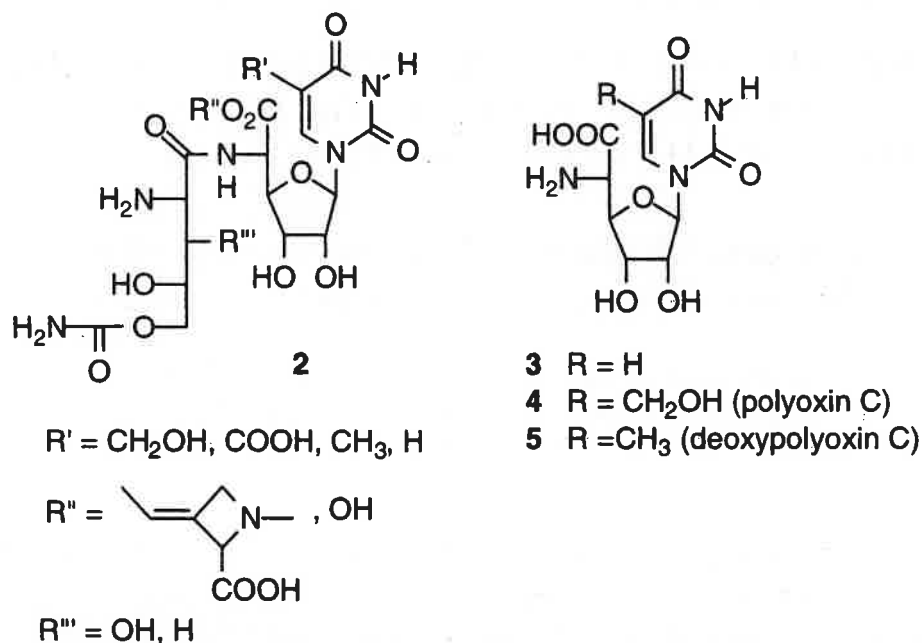
figure 1

We hereby report an efficient de novo synthesis of a 2'-dideoxypolyoxin analogue via a strategy (eq. 1) that permitts stereocontrolled construction of all three contiguous asymmetric centers.

2'-dideoxypolyoxin C analogue

eq. 1
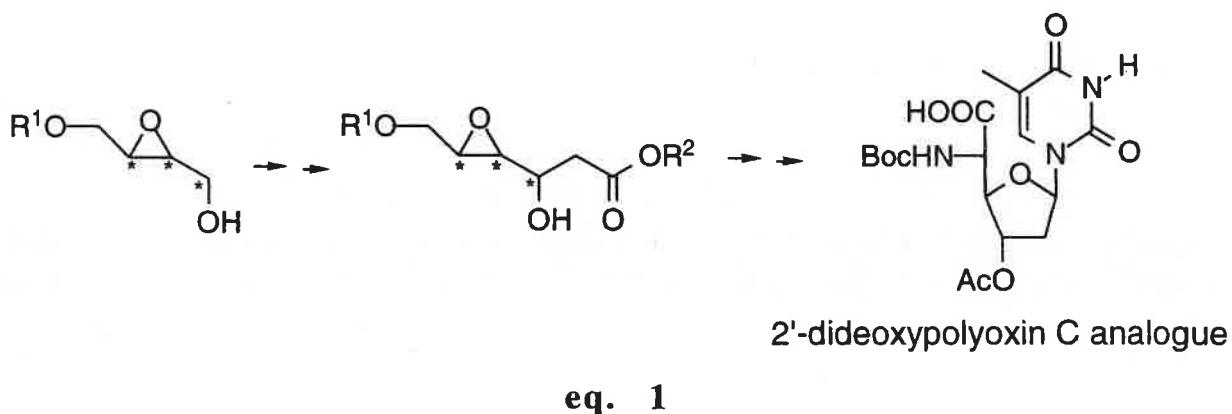
(2S,3S)-4-(Tertbutyldiphenylsilyloxy)-2,3-epoxybutan-1-ol[6] was oxidised via the Doering procedure[7] to the aldehyde which was subjected to the aldolisation reaction. Condensation with lithium tert-butyl acetate at -78 °C leads to the two diastereoisomers *anti* and *syn* in a diastereoisomeric ratio of 75:25 in favour of the desired anti aldol adduct[6] (scheme 1).

When compound **5** reacted under non chelating conditions in presence of 5 eq. of sodium azide and 2.5eq. NH_4Cl in $MeOH/H_2O$ (8/1) four compounds were isolated in 65% total yield.

a) 2 eq iPr$_2$NH, 2 eq nBuLi, 2 eq CH$_3$COOtBu, Et$_2$O, -78 °C to 0°C, 1h b) 5 eq NaN$_3$, 2,5 eq NH$_4$Cl, MeOH/H$_2$O 8/1, reflux 48 h c) TFA, 5 min d) 1,3 eq DIBAH, PhCH$_3$, -78 °C then 8 eq Ac$_2$O, pyr. e) silylated thymine, 1,2 eq CF$_3$SO$_3$SiMe$_3$, ClCH$_2$CH$_2$Cl/CH$_3$CN 1/1, 0°C f) Pd/C 10 %, H$_2$, EtOH/H$_2$O 9/1 f)1,5 eq Boc$_2$O, CH$_2$Cl$_2$, 5 h g) 8 eq HF/pyr., THF/pyr. i) 5 eq pyr.-SO$_3$, 5 eq Et$_3$N, DMSO, CH$_2$Cl$_2$ j) 3 eq NaClO$_2$, 6eq isoamylene, 1 eq K$_2$HPO$_4$, tBuOH, THF, H$_2$O

Scheme 1.

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Compounds **7**, **8** and **9** are issued from a C5 ring opening of the epoxide, while compound **10** is obtained by a C4 nucleophilic attack of the oxirane ring. The observed regioselectivity of the reaction is 98/2 in favour of the C5 ring opening of the epoxide. Tert-butyl and methyl esters **8**, **9** were quantitatively transformed in presence of trifluoroacetic acid to the sole azido-lactone **7** which is one of our key chiral synthons in our synthetic strategy.

Reduction of the azido lactone with diisobutyl aluminium hydride at -78 °C, followed by acetylation (Ac_2O/pyridine) led to a mixture of the corresponding di-acetyls α- and β-furanosides **11** in 95% yield (for the two steps) and an α/β ratio of 40/60. Glycosidation of the 5-methyl pyrimidine derivative **12** with **11** under the conditions developed by Vorbrüggen and co-workers,[8] gave a mixture of the expected nucleosides **13** (76% yield) in a 27/73 α/β ratio.

Catalytical hydrogenolysis of the azide function (Pd/C, EtOH, H_2O) of a mixture of α,β anomers **13** afforded the amino compound **14** in 48% yield. The 5'-amino-2',5'-dideoxynucleoside was converted into the Boc protected derivative **15** which was then treated with 8 eq. of HF/pyridine complex in THF/pyridine to afford the easily separable α,β anomeric monoalcohols **16'** and **16** respectively in 80% yield (2 steps). Selective oxidation of the alcohol **16** to the corresponding aldehyde via the Doering procedure and then oxidation of the crude product led to compound **17** in 40% yield (2 steps).

Conclusion.

A protected analogue of the thymine 2'-polyoxin C was synthetized in 8 steps and an overall yield of 15% starting from the optically active epoxyester **5**. The methodology presented here has the advantage of controlling all asymmetric centers encountered in the molecule and can thus give access to any epimer of compound **17**. Suitably functionalised lithium ester enolates (NH_2, F groups), will also be used for providing a range of C2' modified nucleosides.

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Molecular Cloning of the Gene Encoding Chitosanase from *Bacillus amyloliquefaciens* UTK

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Abstract

The gene encoding a chitosanase from *Bacillus amyloliquefaciens* UTK was cloned into the *Hind*III site of pUC119 and expressed in *Escherichia coli* DH5. The recombinant plasmid contained a 2.0 kb *Hind*III insert of which was sufficient for the expression of chitosanase activity in *E. coli* DH5. Nucleotide sequencing of this region revealed an open reading frame of 837 bp. The protein deduced from this sequence was composed of 278 amino acids. The deduced amino acid sequence from amino acids 1 through 17 coincided with the amino-terminal sequence of the chitosanase, purified from culture of *Bacillus amyloliquefaciens* UTK. The sequence from amino acids 26 to 46 of CSN-UTK exhibited 62 % identities to that of the CSN-N174 and 59 % identities to that of the CSN-MH-K1. This chitosanase was thought to belong to family 46.

Chitosanase (EC 3.2.1.132) is defined as an enzyme that catalyzes random hydrolysis of β-1,4-linkages between *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) residues in a partially *N*-acetylated chitosan. Chitosanase was distinguished from chitinase on the basis of its ability to hydrolyze GlcN-GlcN. Chitosanase can be defined as the enzyme which requires at least one GlcN residue at the either side of hydrolyzing linkages in partially *N*-acetylated chitosan but not GlcNAc-GlcNAc bonds. Chitosanases were subdivided into three subclasses, characterized by the ability to split GlcN-GlcN and GlcNAc-GlcN linkages (subclass I) ; only GlcN-GlcN linkages (subclass II) and GlcN-GlcN and GlcN-GlcNAc linkages (subclass III) [1].

The chitosanases can be classified into two families (Family 46 and Family 8) according to amino acid sequences [2]. Nucleotide sequences and deduced primary amino acid sequences are available for the family 46 chitosanase from *Bacillus circulans* MH-K1 [3],

Streptomyces sp. N174 [4] and *Nocardioides* sp. N106 [5]. Comparison of the three amino acid sequences revealed a significant homology between the N-terminal ends of the mature proteins (amino acids 59-107 from the *B. circulans* chitosanase (CSN-MH-K1) precursor; amino acids 41-90 from the *Streptomyces* sp. N174 chitosanase (CSN-N174) precursor, and amino acids 42-91 from the *Nocardioides* sp. N106 chitosanase (CSN-N106) precursor).

However, the lack of homology in the middle and C-terminal segments between the CSN-MH-K1 and CSN-N174 raises several new questions. Can these two chitosanases be considered as the first members of two distinct subfamilies ? Is there any relationship between these sequence-defined subfamilies and the subclasses on the basis of their cleavage specificity ? To answer these questions, more chitosanase genes and enzymes should be available for molecular and enzymological studies. In this paper, we described a new chitosanase protein and its encoding gene from *Bacillus amyloliquefaciens* UTK, isolated from soil.

Materials and Methods

Bacterial strains and cloning vectors. *Bacillus amyloliquefaciens* UTK was used as the source of the gene for chitosanase. *Escherichia coli* DH5 (supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1) were used as hosts for cloning and sequencing. Plasmid pUC119 was used as the vector.

Cloning of the chitosanase gene from *Bacillus amyloliquefaciens* UTK. Genomic DNA from *B. amyloliquefaciens* UTK was prepared according to the method of Saito and Miura [6]. Genomic DNA and plasmid DNA were both digested with *Hind* III (Takara Shuzo) and ligated with T4 DNA ligase (Takara Shuzo). The ligation mixture was used for transformation of competent *E. coli* DH5 cells. Transformants that appeared on chitosanase detection agar plates (LB agar contained 0.5 % (W/V) colloidal chitosan (degree of acetylated 98 %)) supplemented with ampicillin were determined the production of the chitosanase activity by chitosan degradation zone formation.

Nucleotide sequencing. Restriction fragments were subcloned in an appropriate position of the multiple cloning site of pUC119. The nucleotide sequence was determined by the dideoxy chain termination method of Sanger et al. using a fluorescent dye primer and a modified T7 DNA polymerase (Sequenase, United States Biochemical), on an automated DNA sequencer (Applied Biosystems). Approximately 300 bases were read from a sample in each lane, and both strands of the chitosanase gene were completely sequenced.

Results and discussion

Cloning of the gene for chitosanase

Among several thousand ampicillin-resistant transformants of *E. coli* DH5, one was found to produce chitosanase activity. This chitosanase-expressing transformant contained a 5.2 kb recombinant plasmid (pUC-*csn*). *E. coli* DH5 harboring pUC-*csn* produced extracellular chitosanase activity at a rate of approximately 39 kU per litter of medium, when cells were grown at 37 °C for 10 days. The plasmid encoded chitosanase was active from pH 2.0 to 10.0 with maximum activity at pH 5.0, and it was active from 10 to 60 °C with maximum activity at 50 °C. These characteristics of the activity correspond to those of chitosanase secreted by *B. amyloliquefaciens* UTK (CSN-UTK).

To clarify the origin of the 2.0 kb insert of pUC-*csn* hybridization analysis was done using the digoxigenin-labeled 2.0 kb insert of pUC-*csn* as probe. The labeled insert of pUC-*csn* hybridized with 2.0 kb fragment in *Hind* III digests of the genomic DNA from *B. amyloliquefaciens* UTK blotted on a membrane. Thus, 2.0 kb *Hind* III insert in pUC-*csn* appeared to have been derived from the genomic DNA of *B. amyloliquefaciens* UTK.

Nucleotide sequence analysis

The nucleotide sequence of the 2.0 kb fragment was determined. Fig. 1 shows the nucleotide sequence of a 1200 bp DNA fragment

Fig. 1. Nucleotide sequence of the gene for chitosanase from *Bacillus amyloliquefaciens* UTK and the deduced amino acid sequence of the encoded protein. The nucleotide sequence of the non-transcribed strain is given from 5' to 3', and the deduced amino acid sequence is shown under the nucleotide sequence. The putative ribosome-binding site is shown in box. Horizontal arrows indicate terminator-like, inverted-repeat sequences. The deduced amino acid sequence identical to the amino-terminal sequence of the CSN-UTK protein is indicated by a line under the amino acid sequence.

containing the *csn*-UTK gene. Only one large open reading frame (ORF), beginning with an ATG codon at nucleotide 165 and ending with TAA codon at 1001, was identified in the 837 bp nucleotide sequence that was determined. Upstream from this ORF, there is a putative ribosome-binding site with a sequence of tGAAAGGgGt, followed eight bases later by a potential initiation codon, ATG.

A sequence, resembling the consensus sequence of promoter, was found upstream from the putative ribosome-binding site of the gene from *B. amyloliquefaciens*UTK. This sequence begins at nucleotide 119; it includes the potential -35 region of TCATGTATAC; and the potential -10 region of TACATA;and the two regions are separated by 14 nucleotides.

A large inverted-repeat sequence was found downstream from the termination codon of the ORF (from nucleotides 999 to 1001). However, there is no poly(T) segment downstream from the stem.

Amino acid sequence analysis

The ORF in the nucleotide sequence encoded 278 amino acid residues, as indicated under the nucleotide sequence in Fig. 1. The deduced sequence from amino acids 1 to 17 is identical to the amino-terminal 17 amino acid residues of the chitosanase secreted by *B. amyloliquefaciens* UTK, and the sequence from amino acids -3 to -1 (Val-Phe-Ala) resembles the recognition site of signal peptidases. The chitosanase in *B. amyloliquefaciens* UTK may possibly be cleaved by signal peptidase at the bond between Ala -1 and Ala 1 during secretion across the cytoplasmic membrane.

Homology of the nucleotide sequence

The nucleotide sequence of the *csn*-UTK gene is 83 % homologous to the DNA segment of the chromosomal DNA fragment located around 233° on the *Bacillus subtilis* genetic map (*Bacillus subtilis* 168 genome)[7]. However, no homology could be detected between the CSN-UTK and other chitosanases.

Homology of the amino acid sequence with other enzymes

The deduced amino acid sequence of the CSN-UTK was compared with those of enzymes, reported to date. The sequence from amino acids 26 to 46of CSN-UTK exhibited 62 % identities to that of the CSN-N174 (Fig. 2). In addition, 59 % identities in terms of amino acid sequence was observed between the CSN-UTK and the CSN-MH-K1. Henrissat et al.[2] compared the amino acid sequences of glycosyl hydrolases from various origins by means of hydrophobic cluster analysis[8]. According to their criteria, CSN-UTK seems to belong to

B. amyloliquefaciens UTK IAGINKDQ KRRAEQ TSIFEN GKTET QYBYVI ALDD RGYTCCRAC

Streptomyces sp. N174 AGAGLD HKEIAMEI S EN I W AQYIY LDIGD RGYICC G

Nocardioides sp. N106 AAVGLD HKDIAMEI S EN I W SQYIY EDIKD RGYIAG G

Nocardioides sp. K-01 AIAGLD V KDIAMG S EN I W PQYIY EDIDD RGYIAG G

Amycolatopsis sp. CsO-2 AYPI D A KEIAMEI S EN I W AQYIY EDIGDXRKYIXX X

B. circulans MH-K1 NNTGLDGEQWNNI MKLINKPEQDDLNWIKYYSYCEDIEDERGYTIGLFC

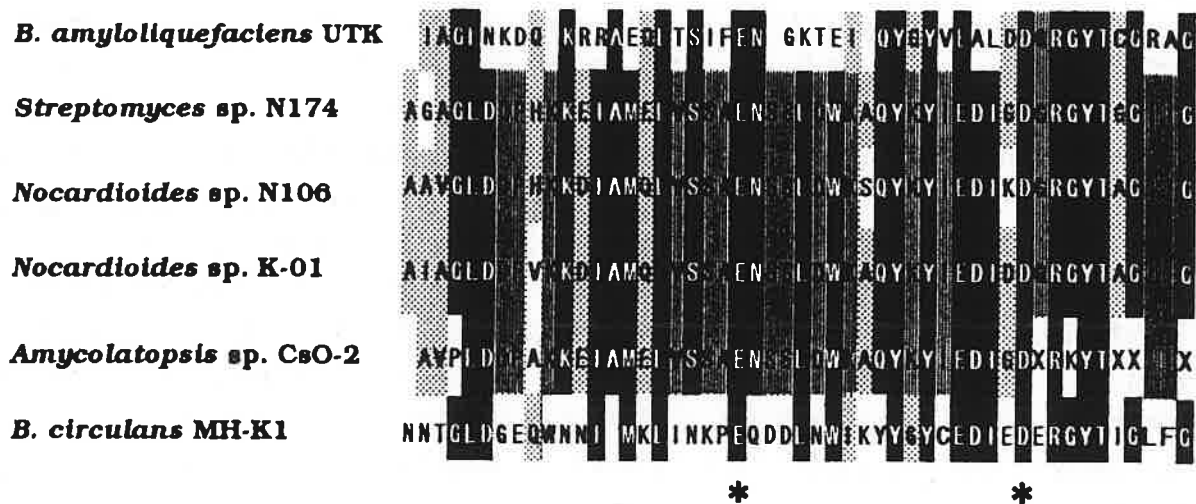   *   *

Fig. 2. Multiple sequence alignment of N-terminal ends of chitosanases (family 46)

family 46, because the CSN-N174 and CSN-MH-K1 have been classified as belong to this family. The only segment that clearly homologous in all family 46 chitosanases is the previously identified N-terminal module. However, there is no homology in the middle and C-terminal segments between the CSN-UTK and the CSN-MH-K1. In comparison with CSN-N174, there is no homology in the C-terminal segment. These three chitosanases may be considered as the first members of three distinct subfamilies. In amino acid sequence, MH-K1 type chitosanase have been found in several *Bacillus* and *Acinetobacter*. N174 type have been found in *Streptomyces*, *Nocardioides*, and *Bacillus*. UTK type have been found in *Bacillus subtilis*. Is there any relationship between these sequence-defined subfamilies and the subclasses proposed by Fukamizo et al. on the basis of their cleavage specificity? From the structure of the oligosaccharide products, it is concluded that CSN-UTK can split the GlcNAc-GlcN and GlcNAc-GlcN linkages in the chitosan molecule (data not shown). The specificity of CSN-UTK is similar to that of *Streptomyces* N174 chitosanase which produces hetero-oligo-saccharides with GlcNAc at the reducing end. These chitosanases should belong to an identical subclass of the specificity (subclass I). The amino acid sequence (amino acids 26 to 199) of chitosanase from UTK is 53% homologous to the chitosanase from N174. In spite of the chitosanases derived from the same genus, the specificity of CSN-UTK is different from that of CSN-MH-K1, which splits the linkages of GlcN-GlcN and GlcN-GlcNAc in the chitosan molecule [9]. CSN-MH-K1 should belong to subclass III.
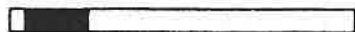
< Family 46 >

Streptomyces sp. N174   subclass I

B. amyloliquefaciens UTK   subclass I

B. circulans MH-K1   subclass III

< Family 8 >

Bacillus sp. No.7-M   subclass II

Bacillus circulans WL-12
endo-1,3-1,4-β-glucanase   subclass III
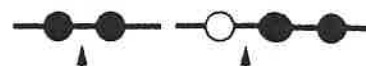
● : GlcN   ○ : GlcNAc

Classification of Chitosanase

Conclusion

The chitosanase from *Bacillus. amyloliquefaciens* UTK belongs to family 46. With the addition of the chitosanase from *Bacillus. amyloliquefaciens* UTK, family 46 of glycosyl hydrolases becomes better defined, including at least three members with high (over 75 %) homology at the nucleotide level. (MH-K1 type, N174 type and UTK type chitosanase)

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Total Sequence of a Bacterial Gene Encoding Chitosanase-Glucanase Activities

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Abstract

Culture supernatant of *Bacillus* sp. (strain D-2) hydrolyzes chitosan and CM cellulose. A 37 kDa protein exhibiting both chitosanase and ß-1,4-glucanase activities was purified from the supernatant by combination of ion-exchange and gel-permeation chromatography. Amino terminal sequence of this enzyme was determined as AGEMMPFPQQV. Using an oligonucleotide primer based on this sequence, a 3031 bp fragment was cloned from the bacterial genome and corresponding amino acid sequence was deduced after its nucleotide arrangement. The open reading frame is 2391 bp long and codes for an 85610 Da protein composed of 797 amino acid residues including a signal peptide. This protein has the typical "family 8" domain conserved in bacterial ß-1,4-glucanases but is lacking in a region proper to genuine chitosanase. It is highly probable that the bacterial family 8 glucanases are accompanied with chitosanase activity.

Keywords: Chitosanase, ß-1,4-glucanase, nucleotide sequence, family 8, bacterial gene

Materials and methods

Strain D-2 of *Bacillus* sp. was isolated from soil of Fukui, Japan. D-2 chitosanase (glucanase) was purified from the culture supernatant by chromatography on SP-Sephadex C-50, Bio-Gel A and DEAE Toyopearl columns. N-terminal sequences were determined using a Procise 492 system (Applied Biosystems). The chitosanase (glucanase) gene was isolated from D-2 genomic DNA, using a LA PCR *in vitro* cloning kit. DNA sequence was determined with Hitachi SQ-5500 and ABI 373A sequencers.

Results and discussion

Strain D-2 of *Bacillus* sp. grown in a liquid medium supplemented with chitosan exhibited extracellular glucanase activity, in addition to chitosanase function, like *Myxobacter*

AL-1[1]. When grown on plates containing chitosan or carboxymethyl cellulose (CMC) and then stained with Congo Red , distinct lytic zones were discerned around the colonies. In order to characterize the enzyme activities, the culture supernatant was concentrated by ammonium sulfate precipitation, dialyzed, and subjected to SDS-polyacrylamide gel electrophoresis in the presence of glycol chitosan or CMC. The gel plates were stained with Coomassie Blue or forwarded to activity staining, after renaturation. As seen in Fig. 1, many protein species were contained in the supernatant, and several of them degraded both glycol chitosan and CMC.

Fig. 1. PAGE profile of chitosanase-glucanase in D-2 culture supernatant and *E. coli* XL1-Blue harboring recombinant plasmid.

SDS-PAGE with CM-cellulose

CM-cellulose

Glycol chitosan

Activity staining
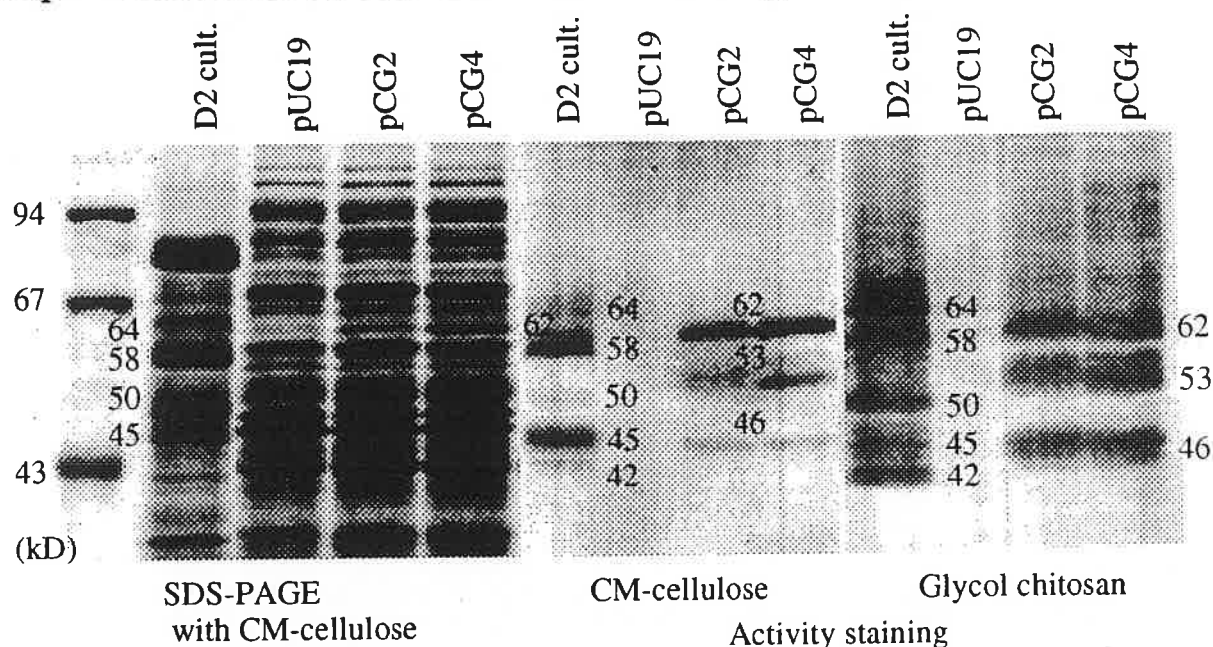
The protein responsible for the chitosanase activity was purified from the culture supernatant, by chromatography using various columns. The purified preparation still contained three protein bands in SDS-PAGE. A 37 kD band retained chitosanase activity, whereas major band (67 kD) was inactive. The two bands were excised, transferred to PVDF membrane and N-terminal amino acids sequences were determined. The sequence, AGEMMPFPQQV, was found to be identical between the inactive 67 kD band and the active 37 kD fragment, suggesting that the active fragment was derived from the inactive 67 kD precursor by proteolysis of the C-terminal region. Based on the 11 amino acids sequence, an oligonucleotide primer was synthesized and entire region of the chitosanase gene was cloned from D-2 genomic DNA. The gene was inserted into pUC19 and electro-introduced into *E. coli* XL1-Blue. Colonies of the *E. coli* bearing the plasmid pCG4

(inserted in series with plac) and pCG4 (in reverse direction) formed lysis-zone on the plate containing colloidal chitosan or CMC, as manifested by Congo Red staining. In addition, sonic extracts from the transformants exhibited distinct chitosanase and ß-1, 4-glucanase activities. The activities were not particularly altered by orientation of the insert or addition of IPTG, indicating that its own promoter was functioning in E. coli.

The nucleotide sequence and the amino acid residues deduced from it is shown in Fig. 2. The sequenced region (3031 bp) contained single open reading frame (2391 bp) encoding for a 797-amino acid precursor polypeptide (the molecular weight was 85610 daltons). Upstream the ORF, putative promoter region was identified: -35 (nucleotide 267 to 272) and -10 (nucleotide 289 to 294) consensus sequences, separated by 16 nucleotides. Shine-Dalgarno sequence was located 9 bp preceding the initiation codon. The N-terminal 11 amino acids of 67 and 37 kD fragments completely coincided with the 42 to 52 amino acid residues of the ORF. Preceding the N-terminus, 41 residues were connected, corresponding to the signal peptide sequence for secretion. The ORF contains the sequence ATDGDLDIAYSLLLAHKQW typical of family 8 glycosyl hydrolases[2], but lacking in significant homology with genuine chitosanases. An inverted repeat was found at the nucleotide 2314 to 2321. The ORF was directly followed by a putative transcription terminator (indicated by *). A stem and loop structure for ρ-independent termination was located 45 bp downstream from the ORF (the nucleotide 2777 to 2808). This inverted-repeat sequence was followed by a T-rich segment.

When extract from E. coli cells harboring the D-2 gene was subjected to SDS-PAGE and then activity staining, three major bands (62, 53 and 46 kD) of chitosanase-glucanase were detected, but no activity was found at the molecular weight zones higher than 65 kD. This fact, together with the finding that the inactive 67 kD protein from D-2 culture supernatant retains intact amino terminal, indicates necessity of proteolytic activation. Probably, different proteases are involved in processing or fragmentation of the precursor in D-2 cells and E. coli.

Fig. 2. Nucleotide-and amino acid-sequences of D-2 chitosanase-glucanase

```
         10        20        30        40        50        60
GAAGCAGGCAAGCATAAGCGGGTGAAGGTGAGCACGGGAGCCGGCAACAAACCGGCCATT
         70        80        90       100       110       120
GCGTTATACCGAAGCTTTGGCTTTGCGGACGCGGGCGAAATCGAGATTGCGCCTGGAGTC
        130       140       150       160       170       180
AGACTTGCCCTGTTCGAGCTGCCCGCGATTCGACAATAGAGCTGAACAATAGCGAAATAA
        190       200       210       220       230       240
```

TAGAGAGTTTGTAGAGTTTAGAAGAAAGTATTTTCATGGGAATCGAAATGTGTTATTTTA
         250   260   270   280   290   300
AAAGCAGGTTTCGAAACCCTATTTTTTTGGCTGTAAAGTTTAAGCGCTTAAACTTAAGGG
                              -35   -10
         310   320   330   340   350   360
CCGAGGTTAGCCAACATCCAATTAAGGAGGCGTTGCAGTTTGTTTACTTCATCGTTATCC
                         S.D.   M F T S S L S
         370   380   390   400   410   420
GGTTCACCGAGATTGAAGTCCGCGTTTCTTCTGCTTCTGTGCTTGGCTATGGTTGTATCG
 G S P R L K S A F L L L C L A M V V S
         430   440   450   460   470   480
ATTGGTTTCGTTCCAAAAGGCAATGAGGGTAGGGTTCACGCCGCCGGCGAGATGATGCCG
 I G F V P K G N E G R V H A A G E M M P
         490   500   510   520   530   540
TTCCCGCAGCAGGTCAGTTATTCCGGCATTATAAAGCCTAACCATGTGACGCAAGCGGCT
 F P Q Q V S Y S G I I K P N H V T Q A A
         550   560   570   580   590   600
ATGAATACAGCCGTAGCGGCCTACTATGATTACTGGAAAGGCAAGTATTTGAAAAACAAT
 M N T A V A A Y Y D Y W K G K Y L K N N
         610   620   630   640   650   660
CTCTCCTCGCTTCCGGGCGGCTACTATGTCAAAGGCGAGATTACAGGCAGTCCTGAAGGC
 L S S L P G G Y Y V K G E I T G S P E G
         670   680   690   700   710   720
TTTGTGCCGCTGGGCACGTCCGAGGGCCAAGGCTACGGGATGATCATTACGGCGCTCATG
 F V P L G T S E G Q G Y G M I I T A L M
         730   740   750   760   770   780
GCCGGACATGATCCCAATGCCCAGACGATCTTCAATGGACTGTTCAAAACTGCACGCGCT
 A G H D P N A Q T I F N G L F K T A R A
         790   800   810   820   830   840
TACAAGAGCTCGGGCAATCCCAATCTGATGGGCTGGGTCGTAGCGGACCATATTAATGCC
 Y K S S G N P N L M G W V V A D H I N A
         850   860   870   880   890   900
CAAGGGCATTTCGGCTCTGCAACGGACGGGGATCTCGACATCGCGTACTCTCTGCTGCTT
 Q G H F G S A T D G D L D I A Y S L L L
         910   920   930   940   950   960
GCCCATAAGCAGTGGGGCTCGAATGGCACGGTCAATTATTTGGCCGAGGCACAAAACATG
 A H K Q W G S N G T V N Y L A E A Q N M
         970   980   990   1000   1010   1020
ATTACGAACGGCATCAAAGCCAGCTATGTGACGACGAACAATCGTCTGAACCTGGGCGAT
 I T N G I K A S Y V T T N N R L N L G D
        1030   1040   1050   1060   1070   1080
TGGGATTCCAAGAGCTCGCTGGCTACGCGTCCTTCCGACTGGATGCTCTCCCATCTTCGC
 W D S K S S L A T R P S D W M L S H L R
        1090   1100   1110   1120   1130   1140
GCTTTCTACGAGTTCACAGGCGATCAGACATGGATCAACGTCATCAACAATCTGTACAAT
 A F Y E F T G D Q T W I N V I N N L Y N
        1150   1160   1170   1180   1190   1200
GTCTATACGCAAGTGAGCAACAACTACGCTTCATCGACAGGATTGATCTCCGACTTCGTC
 V Y T Q V S N N Y A S S T G L I S D F V
        1210   1220   1230   1240   1250   1260

```
GTGAACAATCCTCCGCAGCCTGCACCAGAATGGTACTTGAATGAATTCCAGCAAACGAAT
 V  N  N  P  P  Q  P  A  P  E  W  Y  L  N  E  F  Q  Q  T  N
      1270      1280      1290      1300      1310      1320
GCTTATTATTATAACGCAGCACGTGTGCCGCTCCGCATCGTCATGGATTATGCGATGTAC
 A  Y  Y  Y  N  A  A  R  V  P  L  R  I  V  M  D  Y  A  M  Y
      1330      1340      1350      1360      1370      1380
GGCGATACGCGCGGCAAGACGATCGCAGACAAAATCGCGGTCTGGATTAAAGGCAAAGCA
 G  D  T  R  G  K  T  I  A  D  K  I  A  V  W  I  K  G  K  A
      1390      1400      1410      1420      1430      1440
AGCAACAGTCCAGCTAATATCCGTGACGGCTACCAGTTGAACGGCACGACAATTGGCGGG
 S  N  S  P  A  N  I  R  D  G  Y  Q  L  N  G  T  T  I  G  G
      1450      1460      1470      1480      1490      1500
TACGCAACAGCAGTATTCGTATCGCCGTTCATTGCGGCCAGCACAACCAGCACAAGCCAT
 Y  A  T  A  V  F  V  S  P  F  I  A  A  S  T  T  S  T  S  H
      1510      1520      1530      1540      1550      1560
CAAGCTTGGGTCAATGCGGGCTGGGATTGGATGAAGAACAAGCAGGAGAACTACTTCAGC
 Q  A  W  V  N  A  G  W  D  W  M  K  N  K  Q  E  N  Y  F  S
      1570      1580      1590      1600      1610      1620
GATTCCTATAACCTGATGACGATGCTGTTCATTACGGGCAACTGGTGGAAGCCGACAGCA
 D  S  Y  N  L  M  T  M  L  F  I  T  G  N  W  W  K  P  T  A
      1630      1640      1650      1660      1670      1680
GCTTCAAGCGATACGCAGGCGCCTACGGTGCCGGGCAGCTTAACGGCTGCGGCGACGTCC
 A  S  S  D  T  Q  A  P  T  V  P  G  S  L  T  A  A  A  T  S
      1690      1700      1710      1720      1730      1740
AGCAGCAGTATTAATCTGACTTGGACGGCTTCGACCGACAACGTCGGAGTGACGGGCTAC
 S  S  S  I  N  L  T  W  T  A  S  T  D  N  V  G  V  T  G  Y
      1750      1760      1770      1780      1790      1800
CGCATCTACCGCGGCGGCACTCAGGTGGGCACGGCAACAGGATTGTCTTATGCCGATTCT
 R  I  Y  R  G  G  T  Q  V  G  T  A  T  G  L  S  Y  A  D  S
      1810      1820      1830      1840      1850      1860
GGACTGAGCGCGAATACGAGCTACAGCTATACGGTAAGGGCGGTCGACGCAGCAGGCAAT
 G  L  S  A  N  T  S  Y  S  Y  T  V  R  A  V  D  A  A  G  N
      1870      1880      1890      1900      1910      1920
GTATCCGGCAACAGCAACACAGCTTCTGCAACGACTTTGTCCGGTACGACGCCGCCGACA
 V  S  G  N  S  N  T  A  S  A  T  T  L  S  G  T  T  P  P  T
      1930      1940      1950      1960      1970      1980
GGGACTAATCTGGCCTTGAACAAAACGGCCACCGCAAGCTCGATTGAAGGGGCTGGATTC
 G  T  N  L  A  L  N  K  T  A  T  A  S  S  I  E  G  A  G  F
      1990      2000      2010      2020      2030      2040
GAGGCGTCCAGGGCTTTCGACGGCAGCAGCACAACGAGATGGGCAAGTGCTGAAGGCGTA
 E  A  S  R  A  F  D  G  S  S  T  T  R  W  A  S  A  E  G  V
      2050      2060      2070      2080      2090      2100
GACCCGCAGTGGATCTATGTGAATTTGGGCTCCTCGCAGACCGTCAATCGCGTGAAGCTG
 D  P  Q  W  I  Y  V  N  L  G  S  S  Q  T  V  N  R  V  K  L
      2110      2120      2130      2140      2150      2160
AACTGGGAAGCGGCGTATGCTTCCTCCTATACGATTCAAGTGTCCAATGACAGCGGCACG
 N  W  E  A  A  Y  A  S  S  Y  T  I  Q  V  S  N  D  S  G  T
      2170      2180      2190      2200      2210      2220
CCGACGAATTGGACGACAGTATATACGACAACTACAGGCGACGGAGGCATCGATGACATT
 P  T  N  W  T  T  V  Y  T  T  T  T  G  D  G  G  I  D  D  I
      2230      2240      2250      2260      2270      2280
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ACGTTCACGGCTCGCACGGCCAAGTATGTGCGTGTGCACGGGACGGTCAGAGGCACGCCT
 T  F  T  A  R  T  A  K  Y  V  R  V  H  G  T  V  R  G  T  P
    2290      2300      2310      2320      2330      2340
TACGGGTATTCGCTCTGGGAATTCGAGGTGTACGGGGGCAGTACTGCCCCCTCTAACTTG
 Y  G  Y  S  L  W  E  F  E  V  Y  G  G  S  T  A  P  S  N  L
    2350      2360      2370      2380      2390      2400
GCGCTGAATAAAGCGACAGCGACAAGCTCGATCGAGACAGCAGGCCATGAAGGGGATAAA
 A  L  N  K  A  T  A  T  S  S  I  E  T  A  G  H  E  G  D  K
    2410      2420      2430      2440      2450      2460
GCGGTAGACGGCAATGCAGCAACAAGATGGGCCAGCGCTTACGGCGCATCGCCACAATGG
 A  V  D  G  N  A  A  T  R  W  A  S  A  Y  G  A  S  P  Q  W
    2470      2480      2490      2500      2510      2520
ATTTATATCAATTTGGGCTCGACGCAAAGCATCAGCCGGGTAAAGCTGAATTGGGAAGAT
 I  Y  I  N  L  G  S  T  Q  S  I  S  R  V  K  L  N  W  E  D
    2530      2540      2550      2560      2570      2580
GCATATGCGACCGCTTACTCCATTCAAGTTTCGAATGACAGCGGCTCGACGCCGACGAAC
 A  Y  A  T  A  Y  S  I  Q  V  S  N  D  S  G  S  T  P  T  N
    2590      2600      2610      2620      2630      2640
TGGACGACGGTGTACAGCACGACGACCGGCGACGGCGCTATAGATGACATCACTTTTGCG
 W  T  T  V  Y  S  T  T  T  G  D  G  A  I  D  D  I  T  F  A
    2650      2660      2670      2680      2690      2700
GCGACTAACGCCAAATTCGTTCGTGTCTATGCGACGACAAGAGCGACCGCATACGGATAC
 A  T  N  A  K  F  V  R  V  Y  A  T  T  R  A  T  A  Y  G  Y
    2710      2720      2730      2740      2750      2760
TCGCTTTGGGAATTCGAGGTATACGGAGCTTGATCGCTGGGGCTTGCATGCTTCTCGACA
 S  L  W  E  F  E  V  Y  G  A  *
    2770      2780      2790      2800      2810      2820
TATAGATTGAATTTTGGCTTTGTACGAGGCTGCGGCTTTGTACAGAGCTTTTTTTTTGGG
              >>> >>>>> > >>      << < <<<<<< <<<
    2830      2840      2850      2860      2870      2880
TTTAGAGATGCCTTAATCAGGATATTCATAGTAAGAAGGAGAGTTATCACGTATAATGGA
    2890      2900      2910      2920      2930      2940
ACGTGCGACTTGAAAAGACAAGCATTTGTGACGGAGTGATCGATTGAGTAAAGGAAAAGG
    2950      2960      2970      2980      2990      3000
TAAAGGCGGAACAGGCAGAGGAACGGACAAGAAAGGCTGGAACCGTTGGCAAGCCGGCGC
    3010      3020      3030      3040
TAGACGCGCACAGAACGCCCCCAAGCCTTAC
```

Conclusion

Strain D-2 of *Bacillus* sp. exhibits extracellular chitosanase and ß-1,4-glucanase activities. Gene cloning and nucleotide sequencing demonstrate that single determinant is responsible for both activities. The amino acid sequence deduced from the nucleotide arrangement revealed presence of family 8 domain conserved in bacterial ß-1,4-glucanase proteins.

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An Endo-Chitosanase from *Bacillus* sp. GM44 That Produces Chitosan oligosaccharides with High Degree of Polymerization

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Abstract

For the enzymatic production of chitosan oligosaccharides from chitosan, an endo-chitosanase producing bacterium *Bacillus* sp. GM44 was isolated. The bacterium constitutively produced 50U/ml of chitosanase. The chitosanase was purified from culture supernatant by a CM-Toyopearl column chromatography and Superose 12HR FPLC. Molecular mass of the enzyme was estimated as 45,000 by SDS-PAGE. Its optimum pH and temperature were 5.0 and 70°C, respectively. It was stable in the pH range of 3.0 to 10.0 and up to 40°C. Its N-terminal amino acid sequence had 90% similarity with an endo-cellulase of *Bacillus* sp. KSM-330. It showed very low activity against chitin and CM-cellulose. Chitosan oligosaccharides were fairly resistant to the enzyme action. The enzyme showed maximum activity against a chitosan with 80% deacetylation and showed 83.3% activity against a chitosan with 39.2% deacetylation. It produced chitosan oligosaccharides ranging from chitotriose to chitooctaose as major end-products from chitosan.

Introduction

Chitosan is a β 1,4-linked polymer of glucosamine (GlcN) that occurs only in the cell walls of fungi belonging to the order Mucorales in nature. However, chitosan can be readily obtained by deacetylating chitin that is extracted from an abundant source, shrimp and crab shells. Inspite of their abundance, however, commercial utilization of chitin and chitosan remained neglected for a long time (1, 2). Recently, oligosaccharides obtained from chitosan have received a growing attention because they have a variety of biological activities such as antimicrobial, antitumor, and immuno-stimulants (3-9). Partial acid hydrolysis has been used as a conventional method for obtaining chitosan-oligosaccharides. However, this method requires too much time for fractionation process and gives low yields of oligosaccharides. As an alternative method, enzymatic hydrolysis using chitosanase has been tried by many investigators (10-13). Chitosanase has been found commonly in a variety of microorganisms, including bacteria and fungi. Most of these enzymes catalyze endo-cleavage type reaction.

In this study, we attempted to develop an endo-chitosanase for the production of chitosan oligosaccharides with high degree of polymerization. Here, we have described some properties of an endo-chitosanase purified from *Bacillus* sp. and its

application for the chitosan oligosaccharides production.

Materials and Methods

Isolation and identification of strain GM44

Among the 500 bacterial strains grown on MS agar plates containing chitosan 0.5%, yeast extract 0.5%, K_2HPO_4 0.2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.07%, NaCl 0.05%, KCl 0.05%, $CaCl_2$ 0.01%, and Bacto-agar 1.3% (pH 6.8), strain GM44 was selected as an endo-chitosanase producer. The bacterium was identified by the methods of Bergey's Manual of Systematic Bacteriology.

Purification of endo-chitosanase

Bacillus sp. GM44 was cultivated for 3days in MS broth containing 2% soluble starch as a carbon source in a shaking incubator at 30℃ and 200rpm. After a centrifugation of culture broth (2 ℓ) at 7,000rpm for 10min, supernatant was collected and concentrated with polyethyleneglycol (PEG). The PEG-concentrated enzyme was dialyzed against 10mM Na-acetate buffer (pH 5.0) and loaded on CM-Toyopearl column (2.4×5cm) equilibrated with the same buffer. After washing the column with the equilibration buffer, endo-chitosanase was eluted by 0-0.5M linear gradient of NaCl. The endo-chitosanase fractions were desalted and concentrated with a Centricon and then, subjected to gel permeation chromatography using a Superose 12HR FPLC column.

Endo-chitosanase assay

Endo-chitosanase activity was determined by measuring reducing sugar produced from chitosan. Three hundred microliters of enzyme was mixed with 300 $\mu\ell$ of chitosan dissolved in 50mM sodium acetate buffer (pH5.0) and the reaction mixture was incubated for 10min at 50℃. To stop the reaction and remove undigested chitosan, 10$\mu\ell$ of 10N NaOH was added to the mixture. After a centrifugation, reducing sugars in the supernatant were measured by DNS method (14) with D-glucosamine as a calibration standard. One unit (U) of chitosanase activity was defined as the amount of enzyme that liberated 1µmole of D-glucosamine per min under the described conditions.

Determination of protein and N-terminal amino acid sequence

Protein was determined by the method of Bradford (15) with a bovine serum albumin as a calibration standard. Endo-chitosanase band on the SDS-PAGE gel was transferred to nylon membrane by electro-blotting and the membrane was used for N-terminal amino acid sequencing with a pulse liquid phase protein/peptide sequencer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) and activity staining of endo-chitosanase

SDS-PAGE was carried out by the method of Laemmli (16). After SDS-PAGE, proteins were stained with 0.05% Coomassie brilliant blue R-250. For activity staining, endo-chitosanase was stained as followings. The gel was incubated in 100mM Na-acetate buffer (pH 5.0) containing 1% Triton X-100 for 24hr. After

that, 2.5% agarose gel containing 0.02% chitosan in 10mM Na-acetate buffer (pH 5.0) was overlayed onto the gel and incubated for 1hr at 50℃. Endo-chitosanase band was visualized by staining the agarose gel with 0.1% Congo red.

Analysis of chitosan oligosaccharides

The reaction end products of endo-chitosanase were qualitatively analyzed by thin layer chromatography (TLC) using a silica gel plate (Kieselgel 60 F254, Merck). After developing the products with a solvent system of n-propanol-30% ammonia water (2:1, v/v), the spots were visualized by spraying 0.1% ninhydrin in n-butanol saturated water and baking in a 110℃ oven for 10min. For quantitative analysis, the reaction products were analyzed by HPLC on a TSK-Gel NH_2-60 column with a solvent of acetonitrile-water(60:40, v/v) as an eluant at a flow rate of 1㎖/min.

Results and Discussion

Isolation and Identification of strain GM44

Among the 500 bacterial isolates, strain GM44 was selected as an endo-chitosanase producer. The bacterium produced a high level of endo-chitosanase, about 50U/ml that hydrolyzed chitosan into oligosaccharides ranging from trimer to octamer. Strain GM44 belonged to the genus *Bacillus*. However, the more systematic studies are required for the species identification of strain GM44. We tentatively classified strain GM44 as *Bacillus* sp.

Purification of endo-chitosanase

Because *Bacillus* sp. GM44 produced endo-chitosanase constitutively, the bacterium was cultivated in MS broth containing 2% soluble starch as a carbon source. After cultivation, endo-chitosanase was purified from the culture supernatant by a concentration with PEG, CM-Toyopearl chromatography, and Superose 12HR FPLC. Summary of the purification steps is shown in Table 1. Purified enzyme had a specific activity of 1,744.3U/㎎ and showed single endo-chitosanase band with a molecular weight of 45,000Da (Fig. 1).

Table 1. Purification steps of endo-chitosanase from *Bacillus* sp. GM44.

Step	Total protein (mg)	Total activity (U)	Yield (%)	Purification (-fold)
Culture supernatant	161.8	11,660	100.0	1.0
PEG concentration	81.8	105,48	90.5	1.8
CM-Toyopearl	7.5	8,498	72.9	15.8
Superose 12HR	3.1	5,429	46.5	24.2

Properties of endo-chitosanase

Enzymatic properties of endo-chitosanase are summarized in Table 2. The endo-chitosanase showed higher activity toward long-chained polysaccharide chitosan rather than short-chained oligosaccharides. Against various deacetylated chitosan

Table 2. Properties of an endo-chitosanse purified from *Bacillus* sp. GM44.

Test	Property
Molecular weight	45,000 by SDS-PAGE
N-terminal amino acid sequence	A-X-X-K-E-M-K-P-F-P-Q-Q-V-N-N-Y-A-G-V-I-K
Optimum pH	5.0
pH stability	pH 3.0 ~ 10.0
Optimum temperature	70 °C
Temperature stability	Stable after 16hr-incubation at 40 °C
Effect of metal ions	Inhibited by 1mM of Ag^{2+} or Hg^{2+}
Substrate specificity	Higher activity against chitosan than oligosaccharides. Very low activity against swollen chitin and CM-cellulose
Effect of the degree of deacetylation	Against deacetylated chitosan(40-95% deacetylation), the activity ranges between 70-100%
Reaction products from chitosan	Mainly chitotriose to chitooctaose.

(40-95% deacetylation), the activity ranged between 70-100%. The endo-chitosanase hydrolyzed chitin and CM-cellulose with very low activities, less than 3% of maximum activity. But its N-terminal amino acid sequence had 90% homology with
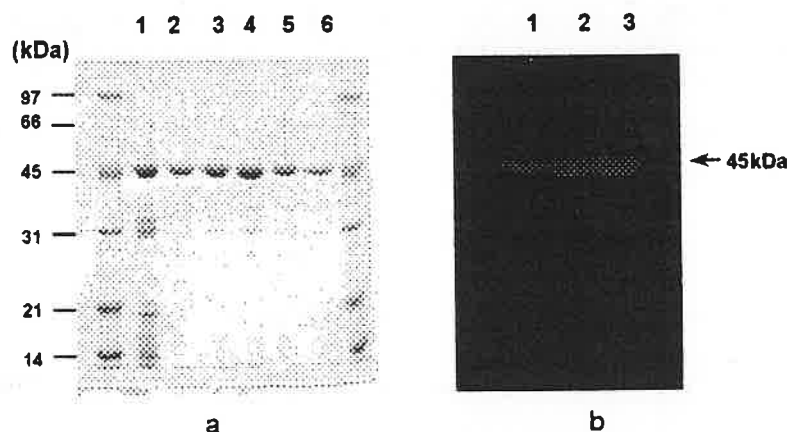

Fig. 1. SDS-PAGE of purified chitosanase from *Bacillus* sp. GM44. (a) Coomassie staining. Lanes: S, standard marker proteins; 1-6. active fraction of Superose 12HR FPLC. (b) Activity staining. Lanes: 1. culture supernatant; 2. CM-Toyopearl ion exchange chromatography; 3. Superose 12HR FPLC.

an endo-cellulase of *Bacillus* sp. KSM-330(17), suggesting that both enzymes have a structural and functional similarity. Time-courses of chitosan hydrolysis are shown in Fig. 2. When 4g chitosan dissolved in 100ml of 2% acetic acid was treated with 20-40U of endo-chitosanase, hydrolysis reached a plateau within 15hr. Oligosaccharides composition of the chitosan hydrolysates was in range of chitotriose to chitooctaose (Fig. 3).
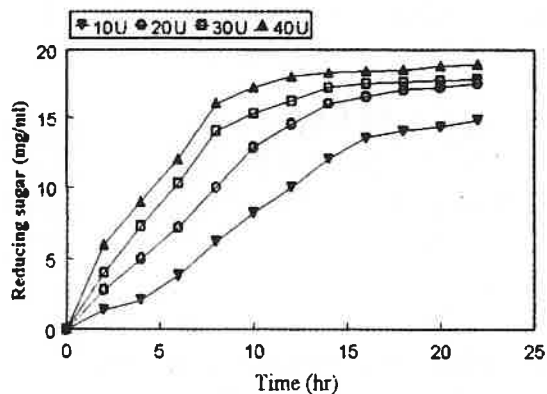
Fig. 2. Time course of chitosan hydrolysis with *Bacillus* sp. GM44 endo-chitosanase. Four g of chitosan dissolved in 100mℓ of 2% acetic acid was hydrolyzed with different amounts of endo-chitosanase.
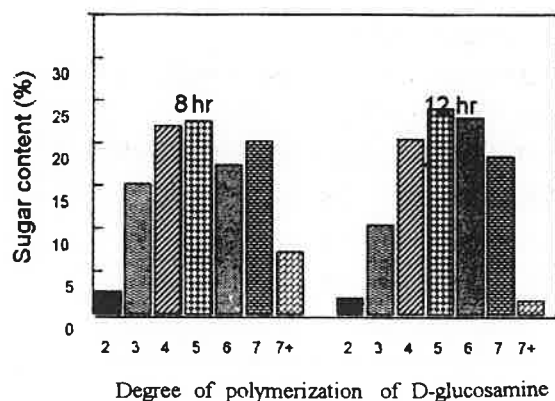

Fig. 3. Oligosaccharides composition of chitosan hydrolysates. Four g of chitosan dissolved in 100mℓ of 2% acetic acid was treated with 20U of endo-chitosanase and the samples taken at 8hr and 12hr were analyzed with HPLC.

Conclusion

In this study, we isolated a *Bacillus* sp. GM44 as an endo-chitosanase producer from soil and investigated some properties of the enzyme purified from the culture supernatant. Our results clearly indicated that the endo-chitosanase could be used for the enzymatic production of the chitosan oligosaccharides with high degree of polymerization ranging from chitotriose to chitooctaose.

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