Some Properties of a Chitinase from a Marine Luminous Bacterium, *Vibrio fischeri* Strain COT-A136

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A chitinolytic, luminous bacterium was isolated from the intestine of a fish, *Oplegnathus tunctatus*, and identified as *Vibrio fischeri*. A chitinase from the culture supernatant of the intestinal luminous strain, *V. fischeri* COT-A136, was purified. The purified chitinase had a molecular weight of 63000, comprising a dimer of 31500 molecular weight subunits. The enzyme was most active at pH 6.0 and 45 °C, and stable below 40 °C. The chitinase had high activities at 2% through 5% NaCl concentration, but the activity was suppressed at low and high NaCl concentrations.

Keywords chitinase; luminous bacterium; marine bacterium; *Vibrio fischeri*

Introduction

Chitinase (EC3.2.1.14) is widely distributed in animals, plants and microorganisms. Microbial chitinases have been purified from cultures of genera such as *Streptomyces*,[1-4] *Serratia*,[5,6] *Vibrio*[7] and *Aeromonas*.[8]

Recently, marine luminous bacteria were isolated from the gut of the fish, *Oplegnathus tunctatus*, in our laboratory, and high extracellular chitinase activity was found in the culture supernatants of most of these luminous strains.

In this paper we describe the purification and some properties of extracellular chitinase from one of these intestinal luminous strains.

Experimental

Materials and Isolation The fish, *Oplegnathus tunctatus*, which was collected in Amami (Okinawa, Japan), was kindly donated by Mr. R. Satoh of Taiyo Central R & D Institutes, Taiyo Fishery Co., Ltd. The isolation of intestinal luminous bacteria was carried out by the method previously described.[9]

Detection of Extracellular Chitinase Activity Extracellular chitinase activity was detected on chitin seawater agar plates that consisted of 10 g of chitin powder (Nacalai Tesque, Kyoto, Japan) per liter and NC agar medium.[10] The NC broth medium lacking agar is usually used as a basal medium. The inoculated chitin seawater agar medium was incubated at 25 °C for 2—7 d. Clear zones indicating chitinolytic activity could be seen around the colonies of extracellular chitinase producing bacteria.

Cultivation for Preparation of Chitinase The culture medium consisted of the basal medium described above and 10 g of chitin powder per liter. Cultivation was carried out at 23 °C for 6 d by the method previously described.[9] After cultivation, the cells were removed from the culture by centrifugation at 12000 × *g*.

Assay of Chitinase Activity The chitinase activity was measured according to the methods of Jeuniaux[11] and Yabuki *et al.*[8] The assay mixture was composed of 1.4 ml of colloidal chitin suspension, 1.4 ml of 0.1 M Tris–HCl buffer (pH 7.8) containing 6% NaCl and 0.2 ml of the enzyme solution. The initial absorbance at 610 nm of the assay mixture was adjusted from 0.5 to 0.8. After incubation for 60 min at 30 °C, absorbance at 610 nm (A_{610}) of the assay mixture was determined with a Shimadzu UV-120-02 spectrophotometer. One unit of activity was defined as the amount of enzyme that caused a 1% decrease in A_{610} per min per ml. The enzyme solution was adjusted to a level such that the decrease in A_{610} did not exceed 20%. *β*-*N*-Acetylglucosaminidase and *β*-*N*-acetylgalactosaminidase activities were measured according to the method of Ohtakara *et al.*[12] Lysozyme activity was measured by the method of Koga *et al.*[13]

Identification Identification of the bacterial isolates was carried out according to the methods of Reichelt and Baumann,[14] Jensen *et al.*,[15] Baumann and Baumann,[16] Yang *et al.*[17] and Baumann *et al.*,[18] as previously described.[19]

Determination of Protein Concentration Protein concentration was determined by the method of Lowry *et al.*[20] with bovine serum albumin as a standard.

Electrophoresis Polyacrylamide disc gel electrophoresis (PAGE) was performed by the method of Davis.[21] Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed by the method of Weber and Osborn.[22]

Measurement of Molecular Weight The molecular weight of the purified enzyme was estimated by SDS-PAGE and gel filtration on a column of Sephadex G-100 superfine (1.5 × 95 cm) by the method of Determann and Michel.[23]

Chemicals Chitin powder was purchased from Nacalai Tesque Inc., Kyoto, Japan. *p*-Nitrophenyl-*β*-*N*-acetylglucosaminide and *p*-nitrophenyl-*β*-*N*-acetylgalactosaminide were obtained from Sigma Chemical Co., St. Louis, U.S.A. Other chemicals were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Results and Discussion

Identification of the chitinolytic, luminous bacterium COT-A136 was carried out. The COT-A136 strain is a moderately luminous, motile gram-negative, rod. It was negative for the traits of accumulation of poly-*β*-hydroxybutyrate, production of gas on fermentation of D-glucose and activity of arginine dihydrolase. The COT-A136 strain grew at 20, 30 and 35 °C, but not at 4 and 40 °C. As sole carbon and energy sources, it utilized maltose, cellobiose and L-proline, but was unable to grow on D-glucuronate, mannitol, D-*α*-alanine, DL-*β*-hydroxybutyrate, acetate, propionate, L-tyrosine, pyruvate, *α*-ketoglutarate, sucrose, salicin, caprylate and pelargonate. The COT-A136 strain did not produce amylase, gelatinase and lipase. These results indicate that the intestinal luminous strain, COT-A136, is a strain of *Vibrio fischeri*.

The purification of the chitinase from *V. fischeri* COT-A136 is summarized in Table I. Ammonium sulfate was added to the culture supernatant (2600 ml) to 100% saturation. The precipitate was dissolved in an aliquot of 10 mM Tris–HCl buffer (pH 7.8) and then dialyzed against 5 mM Tris–HCl buffer (pH 7.8) at 4 °C for 5 d. The dialyzate (1500 ml) was mixed with DEAE-Sephacel equilibrated with 10 mM Tris–HCl buffer (pH 7.8) at 20 °C. The chitinase was eluted with the same buffer containing 0.8 M NaCl and

TABLE I. Summary of Purification of the Chitinase from *V. fischeri* COT-A136

Procedure	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
Culture supernatant	3600	1090	3.3	100
Ammonium sulfate precipitation	2500	310	8.1	69
DEAE-Sephacel	1400	120	11.7	39
Sephadex G-100	950	68	14.0	26

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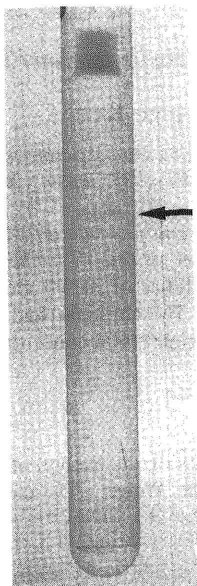

Fig. 1. PAGE of the Purified Chitinase

Electrophoresis was done using 10% gel as described in Experimental. Protein was stained with Coomassie Brilliant Blue R-250.

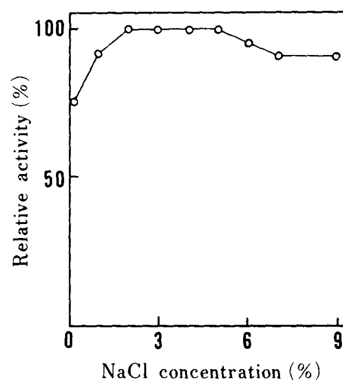

Fig. 2. Effect of NaCl Concentration on the Activity of the Purified Chitinase

3 M urea, because little enzyme was eluted with the buffer containing 0.8 M NaCl or the buffer containing 0.8 M NaCl and 1 M urea. On the other hand, *β-N*-acetylglucosaminidase and *β-N*-acetylgalactosaminidae activities were detected in the fractions eluted with the buffer containing 0.2 M NaCl.

The active fractions of chitinase were combined and then applied to a Sephadex G-100 column (3.0×45 cm) equilibrated with the same buffer at 4 °C. The chitinase was eluted as a single peak and then collected. No lysozyme activity was detected in this fraction.

The purified chitinase had a specific activity of 14.0 units per mg of protein and showed 4.2-fold purification over the original culture supernatant, with 26% recovery. The purified enzyme showed a single protein band on PAGE (Fig. 1) and SDS-PAGE. After electrophoresis (PAGE), the gel was sliced in test tubes with the buffer. The chitinase activity was detected in the eluate from the gel corresponding to the stained band.

The subunit molecular weight of the chitinase from *V. fischeri* COT-A136 was estimated to be 31500 by SDS-PAGE. On Sephadex G-100 superfine gel filtration, the molecular weight of the purified enzyme was estimated to be 63000, suggesting that the enzyme is a dimer.

The molecular weights of some microbial chitinases have been reported, *i.e.*, 33000 and 25000 for chitinases I and II of *Streptomyces orientalis*,[3] 60000 for *Vibrio* sp.[7] and 115000 for *Aeromonas hydrophila* subsp. *anaerogenes* A52.[8]

The molecular weight of the chitinase from the COT-A136 was similar to that of *Vibrio* sp.[7]

The chitinase activity of the marine, intestinal luminous bacterium, COT-A136, was affected by NaCl concentration, as shown in Fig. 2. This chitinase exhibited high activity at NaCl concentrations ranging from 3% to 5%, but the activity was suppressed at both low and high NaCl concentrations. Whereas the proteinase from luminous bacteria, *V. splendidus* and *V. logei*, exhibited higher activities at low NaCl concentration[9,19] and the activity of agarase from a luminous strain, *V. harveyi* FLB-17, was suppressed at low NaCl concentration, but not affected from 3% to 8% NaCl.[24] Regarding the effect of NaCl concentration on the enzyme activity, the property of this chitinase was different from that of the proteinases and the agarase.

The optimum pH of the purified chitinase was pH 5.0—6.0. The chitinases from bacteria, *Vibrio* sp.[7] and *A. hydrophila* subsp. *anaerogenes* A 52[8] were most active at pH 6—8 and pH 7. The optimum pH of this chitinase was lower than that of previously reported chitinases.

The purified chitinase was stable below 40 °C and most active at 45 °C, which was similar to that of *A. hydrophila* subsp. *anaerogenes* A52.[8]

The distribution of luminous bacteria in the intestine of the fish, *Oplegnathus tunctatus*, examined in this study was diverse corresponding to intestinal microflora. The ratio of the numbers of luminuos bacteria, which ranged in density from 10^3 to 10^4 cells per centimeter of the intestine, against total intestinal bacteria, that could grow on MN agar (nutritionally complete medium[25]) at 20 °C for 48 h, was observed more than 10% on average.

It may be considered that these enzymes contribute to making up a major population with luminous bacteria in the intestinal microflora.

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References

1) C. Jeuniaux, *Arch. Int. Physiol. Biochem.*, **67**, 597 (1959).
2) J. Skujins, A. Pukite and A. D. McLaren, *Enzymologia*, **39**, 353 (1970).
3) Y. Tominaga and Y. Tsujisaka, *Agric. Biol. Chem.*, **40**, 2325 (1976).
4) S. Hara, Y. Yamamura, Y. Fujii and T. Ikenaka, "Proceedings of the Second International Conference on Chitin and Chitonsan," ed. by S. Hirano and S. Tokura, the Japanese Society of Chitin and Chitosan, 1982, p. 125.
5) J. Monreal and E. T. Reese, *Can. J. Microbiol.*, **15**, 689 (1969).
6) R. L. Roberts and E. Cabib, *Anal. Biochem.*, **127**, 402 (1982).
7) A. Ohtakara, M. Mitsutomi and Y. Uchida, *J. Ferment. Technol.*, **57**, 169 (1979).
8) M. Yabuki, K. Mizushima, T. Amatatsu, A. Ando, T. Fujii, M. Shinada and M. Yamashita, *J. Gen. Appl. Microbiol.*, **32**, 25 (1986).
9) S. Fukasawa, S. Takei, R. Satou, H. Shima and M. Kurata, *Chem. Pharm. Bull.*, **38**, 1644 (1990).
10) S. Fukasawa, P. V. Dunlap, M. Baba and M. Osumi, *Agric. Biol.*

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Chem., **51**, 265 (1987).
11) C. Jeuniaux, "Methods in Enzymology," Vol. 8, ed. by E. F. Neufeld and V. Ginsburg, Academic Press, New York, 1966, p. 644.
12) A. Ohtakara and K. N. T. Thi, *Nippon Nogeikagaku Kaishi*, **52**, 589 (1978).
13) D. Koga, T. Imoto, S. Tanaka, A. Ida and K. Yagishita, *Agric. Biol. Chem.*, **47**, 1941 (1983).
14) J. L. Reichelt and P. Baumann, *Arch. Mikrobiol.*, **94**, 283 (1973).
15) M. J. Jensen, B. M. Tobo, P. Baumann, M. Mandel and K. H. Nealson, *Cur. Microbiol.*, **3**, 311 (1980).
16) P. Baumann and L. Baumann, "The Prokaryotes," ed. by M. P. Starr, H. Stolp, H. G. Trupper, A. Balows and H. G. Schlegel, Springer-Verlag, Berlin, Heidelberg and New York, 1981, p. 1301, Chapter 104.
17) Y. Yang, L. Yeh, Y. Cao, L. Baumann, P. Baumann, J. S. Tang and B. Beaman, *Cur. Microbiol.*, **8**, 95 (1983).
18) P. Baumann, R. H. W. Schubert, A. L. Furniss, J. V. Lee and L. Baumann, "Bergey's Manual of Systematic Bacteriology," Vol. 1, ed. by N. R. Kreig and J. G. Holt, Williams and Wilkins, Baltimore and London, 1984, p. 516.
19) S. Fukasawa, S. Aoki, N. Gawazawa and M. Osumi, *Agric. Biol. Chem.*, **52**, 427 (1988).
20) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
21) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
22) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
23) H. Determann and W. Michel, *J. Chromatogr.*, **25**, 303 (1966).
24) S. Fukasawa and H. Kobayashi, *Agric. Biol. Chem.*, **51**, 269 (1987).
25) S. Fukasawa and P. V. Dunlap, *Agric. Biol. Chem.*, **50**, 1645 (1986).

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