

## Purification and Characterization of Chitinolytic Enzymes Produced by *Aeromonas* sp. J-5003

Yong Un Choi, Ji Hee Kang, Myung Suk Lee and Won Jae Lee\*

Department of Microbiology, Pukyong National University, Busan 608-737, Korea

(Received July 2002, Accepted January 2003)

Chitinase and chitinase produced by *Aeromonas* sp. J-5003 were purified and characterized. The chitinase was purified to 19.4 folds by gel chromatography and ion-exchange chromatography with the overall yield of 2.2% and the specific activity of 93.1 unit/mg. The purified enzyme showed a single band on SDS-PAGE with MW 54 kDa. The optimum pH and temperature of the purified chitinase were 7.0 and 37°C, respectively, and this enzyme stable in the range of pH 6.0 to 10.0 below 37°C. Mg<sup>2+</sup>, Ca<sup>2+</sup> and Na<sup>+</sup> slightly stimulated the chitinase activity. However, Hg<sup>2+</sup> and Fe<sup>3+</sup> inhibited chitinase activity. The chitinase was purified by Sephacryl HR-100 gel chromatography and DEAE-Sephadex A-50 ion-exchange chromatography with 33.5 purification folds and 4.3% yield. The purified enzyme showed a single band with MW 63 kDa. The optimum pH and temperature of the purified chitinase were 7.0 and 37°C, respectively. And this enzyme was stable in the range of pH 6.0 to 9.0 and at the temperature below 37°C. The enzyme activity was increased by Mn<sup>2+</sup>, but it was inhibited by Ag<sup>+</sup>.

Key words: Chitinase, Chitinase, *Aeromonas* sp. J-5003, Purification

### Introduction

Chitin is very abundant materials on earth, which is polymerized by  $\beta$ -1,4 linkage of N-acetyl- $\beta$ -D-glucosamine (NAG). It is often contained within crab shell, insect shell and mollusk epidermis, and produced about 100 billion ton per year. Chitin and its hydrolyzed materials are very effective in decrease of blood pressure, triglyceride and cholesterol in blood, and prevention of weight loss by cancer. It has been also reported that they improved the recovery of disease of adult people and activated the energy metabolism of cell (Kim, 1997).

There are a number of reports that chitinolytic enzyme was detected from invertebrates, fishes, amphibia, reptiles and birds (Jeuniaux, 1996; Lundblad et al., 1975; Ohtakara et al., 1978). In case of plants, chitinase is present in soybeans, wheat germ and yam (Jeuniaux, 1961; Molano et al., 1979; Si and Li, 1970; Wadworth and Zikakis, 1984). But, many valuable industrial chitinolytic enzymes are mainly

obtained from microorganisms. Microbial chitinases were reported in *Penicillium* (Fenton and Eveleigh, 1981), *Saccharomyces* (Molloy and Burke, 1997), *Serratia* (Monreal and Reese, 1969), *Aeromonas* (Huang et al., 1996; Mitsuhiro and Arai, 1992; Yabuki et al., 1986), *Alteromonas* (Hayashi et al., 1995; Tsujibo et al., 1991), *Bacillus* (Bhushan and Hoondai, 1998; Pelletier and Sygusch, 1990; Sakai et al., 1998) and *Pseudomonas* (Kang and Chung, 1999; Lee et al., 1998).

We previously isolated *Aeromonas* sp. J-5003 on the coast of Busan and found that this strain produced two chitinolytic enzymes, chitinase and chitinase (Choi et al., 2003). In this study, we investigated purification and characterization of chitinase and chitinase produced by *Aeromonas* sp. J-5003.

### Materials and Methods

#### Bacterial strains and media

*Aeromonas* sp. J-5003, which produces chitinase and chitinase, had been previously isolated and

\*Corresponding author: wjlee@pknu.ac.kr

characterized (Choi et al., 2003). Preparation of colloidal chitin was performed by Choi et al. (2003). The medium for the production of chitinase was consisted of colloidal chitin 0.5%, yeast extract 0.25%, peptone 0.25%, glucose 0.2%, NaCl 1.5%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, KH<sub>2</sub>PO<sub>4</sub> 0.03%, K<sub>2</sub>HPO<sub>4</sub> 0.07%, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.1%, ZnSO<sub>4</sub> 0.01% and MnCl<sub>2</sub> 0.01%. For chitobiase production, glucose and peptone were replaced by 0.2% galactose and 0.5% tryptone in the above medium.

#### Purification of chitinase and chitobiase

The bacterial preculture was inoculated into the culture medium. Initial pH of the medium was adjusted to 7.0 and it was incubated for 88 hrs at 30°C on a rotary shaker at 210 rpm. The culture was centrifuged for 20 min at 4,000×g using a refrigerated centrifuge to remove the cells. Solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added at 80% saturation into the supernatants. After stirring for overnight at 4°C, the precipitate was collected by centrifugation at 4,000×g, 4°C and resuspended into 20 mM sodium phosphate buffer (pH 7.0; buffer A). The collected precipitates were dialyzed with buffer A, and stored at -70°C. The frozen precipitates were used as crude enzyme preparations for further purification.

40 milliliter of the crude enzyme solution was placed on top of Sephadex G-200 column (2.3×50 cm, Amersham Pharmacia, Sweden) and eluted with buffer A. The fractions containing chitinase activity were collected and concentrated. Concentrated chitinase was subjected to 1.5×15 cm Q-Sepharose column. The column was developed by linear gradient elution into 3.5 ml fractions using 0–1.0 M NaCl at a flow rate of 30 ml/hr. The active fractions were pooled and dialyzed against buffer A for overnight.

Purification of chitobiase was proceeded as follows: The crude enzyme was put on a Sephacryl HR-100 column (2.3×50 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0). Active fractions were collected, then applied to DEAE-Sephadex A-50 column (2.1×30 cm). The elution was performed with 0–1.0 M NaCl linear gradient. The active fractions were collected, and its purity was determined by SDS-polyacrylamide gel electrophoresis (PAGE).

#### SDS-PAGE

Molecular weight and purity of the purified enzymes were assayed by SDS-PAGE, which was performed according to the method of Laemmli (1970) using 8.0% polyacrylamide gel. The gels were stained with Coomassie brilliant blue R-250.

#### Enzyme assay

One milliliter of chitinase solution was mixed with 1.0 ml of substrate solution (1.0% colloidal chitin solubilized in 20 mM sodium phosphate buffer, pH 7.0) and allowed to react at 37°C for 45 min. The reaction was terminated by boiling the reaction mixture at 100°C for 5–10 min, and it was centrifuged. The supernatant was used to determine chitinase activity by dinitrosalicylic acid (DNS) assay (Miller, 1959). One unit of chitinase was defined as the amount of enzyme required to produce the reducing equivalent of 1 μmol of N-acetylglucosamine per min under the assay conditions.

Chitobiase activity was determined with the modified method of Borooah et al. (1961). The reaction mixtures contained 0.1 ml of the purified enzyme solution, 0.2 ml of 5 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate and 0.7 ml of 0.05 M sodium phosphate buffer. The assays were performed for 20 min at 37°C. 2.0 ml of 0.25 M Na<sub>2</sub>CO<sub>3</sub> was added to stop the reaction, and then A<sub>405</sub> was measured. One unit of enzyme activity catalyzes the formation of 1 μmole of *p*-nitrophenol per min.

#### Protein concentration measurement

Protein concentration was measured by the method of Lowry (1951) with bovine serum albumin as standard material.

#### Effect of pH and temperature on the chitinase and chitobiase activity

To measure the optimum pH range for chitinase and chitobiase activity, the enzyme solutions (0.5 ml) dissolved in different pH buffer were added into 0.7 ml of 1.0% of colloidal chitin substrate solution. This mixture was reacted at 37°C for 45 min and each of activity was assayed. Enzyme activity was also measured at various temperatures with pH 7.0.

**Effect of pH and temperature on the chitinase and chitobiase stability**

Two enzymes were preincubated for 1 hr at various pH values and temperatures without the substrates, and immediately the remaining activity was assayed.

**Effect of metal ions on the chitinase and chitobiase activity**

The effect of metal ions on enzyme activity was assayed with MgCl<sub>2</sub>, NaCl, CuCl<sub>2</sub>, HgCl<sub>2</sub>, CaCl<sub>2</sub>, ZnCl<sub>2</sub>, FeCl<sub>3</sub>, AgNO<sub>3</sub>, CdCl<sub>2</sub>, MnCl<sub>2</sub>, PbCl<sub>2</sub> and KCl. Distilled water containing each of the metal ions was added into 0.7 ml of the enzyme solution with final concentration 1 mM and stood for 1 hr at 37°C. The residual enzyme activity was then measured.

**Results and Discussion**

**Purification of chitinase and chitobiase**

Chitinase from *Aeromonas* sp. J-5003 was purified to homogeneity by two successive chromatographies of Sephadex G-200 and Q-Sepharose. Purification procedure of chitinase from the culture supernatant was summarized in Table 1. Chitinase was purified 19.4-fold with a yield of 2.2%.

**Table 1. Summary of the purification of chitinase from *Aeromonas* sp. J-5003**

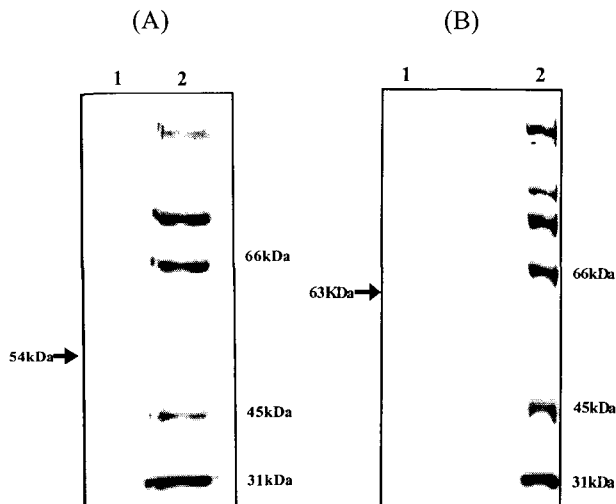
Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Crude supernatant	6,000.0	26,449	4.4	100.0	1.0
Ammonium sulfate precipitation	296.4	7,091.4	23.9	26.8	5.0
Sephadex G-200	14.1	1,234.2	87.5	4.7	18.3
Q-Sepharose	6.2	576.6	93.1	2.2	19.4

Chitobiase from the supernatant was successfully purified by ammonium sulfate precipitation and chromatography. Chitobiase was finally purified to near homogeneity by gel filtration and ion chromatography using Sephacryl HR-100 and DEAE Sephadex A-50 about 33.5-fold with a yield of 4.3% from culture medium. The purification procedure of chitobiase from the culture supernatant was summarized in Table 2. The final preparation gave a

**Table 2. Summary of the purification of chitobiase from *Aeromonas* sp. J-5003**

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Crude supernatant	5,556.6	23,219	4.2	100.0	1.0
Ammonium sulfate precipitation	296.4	7,043	23.8	30.3	5.7
Sephacryl HR-100	12.8	1,368	106.9	5.9	23.1
DEAE-Sephadex 1A-50	7.1	1,000	140.8	4.3	33.5

single band in SDS-PAGE and the molecular weight of chitinase and chitobiase were estimated to be 54 kDa and 63 kDa, respectively (Fig. 1). The purified chitobiase revealed the high value of specific activity and purity, but the recovery rate was low compared to other microbial chitobiases such as those produced by *Penicillium islandicum* (Fenton and Eveleigh, 1981) and *Streptomyces griseus* HUT 6037 (Mitsutomi et al., 1995).



**Fig. 1. SDS-PAGE of chitinase purified from *Aeromonas* sp. J-5003.**

- (A) Lane 1, Chitinase; Lane 2, Molecular size marker.
- (B) Lane 1, Chitobiase; Lane 2, Molecular size marker.

**Effect of pH and temperature on the chitinase and chitobiase activity**

Effects of pH and temperature on chitinase activity were shown in Fig. 2. The highest activity

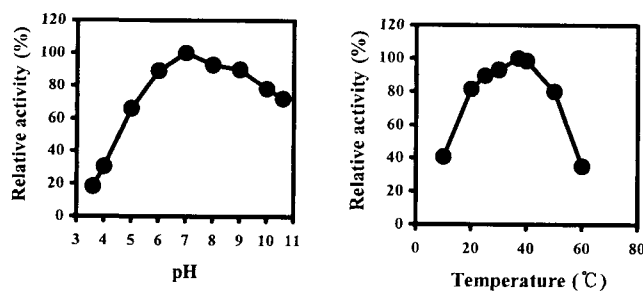


Fig. 2. Effect of pH and temperature on the chitinase activity.

of chitinase was observed at pH 7.0. And even in the wide range of pH 6.0 to 11.0, the enzyme showed activity more than 70%. But, the chitinase activity rapidly decreased below pH 5.0. The highest activity was obtained at 37°C.

Optimum pH and temperature of the chitobiase were 7.0 and 37°C, respectively (Fig. 3). Also the enzyme showed relatively high activity at pH 6.0–9.0 and 25–50°C.

#### Effect of pH and temperature on the chitinase and chitobiase stability

Chitinase produced by *Aeromonas* sp. J-5003 was stable in pH 6.0–10.0 and below 37°C (Fig. 4). The enzyme was inactivated at the acidic pH range and above 37°C.

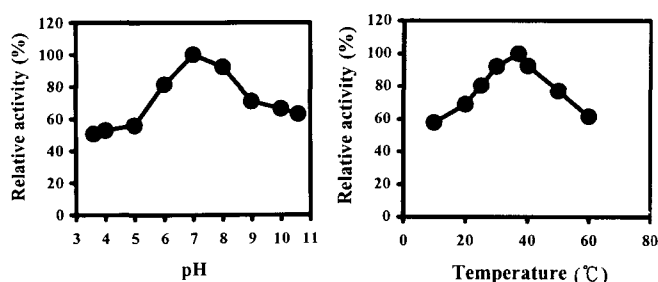


Fig. 3. Effect of pH and temperature on the chitobiase activity.

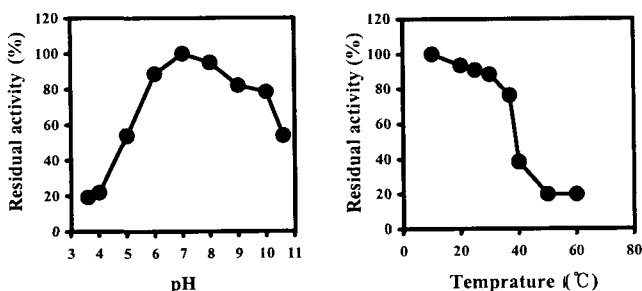


Fig. 4. Effect of pH and temperature on the chitinase stability.

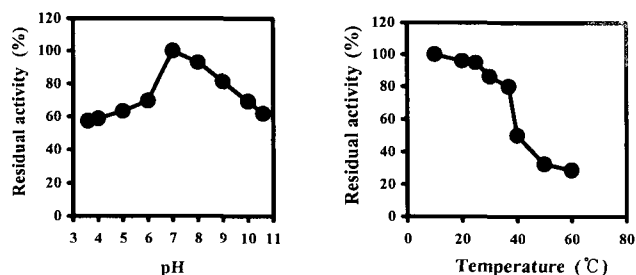


Fig. 5. Effect of pH and temperature on the chitobiase stability.

Chitobiase was stable at the pH 7.0–9.0 (Fig. 5). Heat stability of chitobiase was examined by maintaining the enzyme solution with various temperatures at pH 7.0 for 1 hr. The enzyme activity more than 80% remained after treatment at 37°C, but it was rapidly decreased above 37°C. Yabuki et al. (1986) reported that chitobiase produced by *A. hydrophila* subsp. *anaerogenes* A52 retained 83% of its activity at 45°C, and was inactivated at 55°C. From these results, it is concluded that the chitobiase from *Aeromonas* sp. J-5003 was less heat stable than other microbial chitobiases.

#### Effect of metal ions on the chitinase and chitobiase activity

Table 3 showed effect of metal ions on the chitinase and chitobiase activity. MgCl<sub>2</sub>, CaCl<sub>2</sub> and NaCl increased enzyme activity, but FeCl<sub>3</sub> and HgCl<sub>2</sub> inhibited the enzyme activity. Especially, the

Table 3. Effect of metal ions on the activity of chitinase and chitobiase produced by *Aeromonas* sp. J-5003

Compounds (1 mM)	Chitinase	Chitobiase
	Relative activity (%)	Relative activity (%)
No addition	100.0	100.0
MgCl <sub>2</sub>	103.1	93.8
NaCl	109.9	94.8
KCl	72.0	96.3
AgNO <sub>3</sub>	53.6	65.0
CdCl <sub>2</sub>	57.6	90.2
FeCl <sub>3</sub>	21.6	91.5
MnCl <sub>2</sub>	89.6	104.4
CuCl <sub>2</sub>	66.4	97.9
HgCl <sub>2</sub>	28.1	92.0
CaCl <sub>2</sub>	103.9	90.5
ZnCl <sub>2</sub>	62.4	93.6
PbCl <sub>2</sub>	72.8	95.1

inhibition by HgCl<sub>2</sub> was commonly reported in chitinase-producing microorganisms (Jeuniaux, 1961; Yabuki et al., 1986).

Effect of the various metal ions on the chitobiase activity was also investigated. In case of chitobiase, MnCl<sub>2</sub> slightly increased the enzyme activity, but other metal ions had no marked effect on enzyme activity, with exception of AgNO<sub>3</sub>. These results showed that chitinase and chitobiase from *Aeromonas* sp. J-5003 behaved differently against individual compounds.

Chitinases were produced by various microorganisms including *Saccharomyces* (Molloy and Burke, 1997), *Serratia* (Monreal and Reese, 1969), *Aeromonas* (Huang et al., 1996; Mitsuhiro and Arai, 1992; Yabuki et al., 1986), *Alteromonas* (Hayashi et al., 1995; Tsujibo et al., 1991), *Bacillus* (Bhushan and Hoondai, 1998; Pelletier and Sygusch, 1990; Sakai et al., 1998), *Vibrio* (Bassler et al., 1991) and fungi (Fenton and Eveleigh, 1981). It has been reported that affinity adsorption procedure was effective to purify chitinase (Jeuniaux, 1996; Yabuki et al., 1986). We also tried to use the same procedure to purify chitinase from a crude supernatant of *Aeromonas* sp. J-5003, but it was difficult to use the procedure because of low affinity. So, we purified this chitinolytic enzymes to homologous state by ion chromatography and gel filtration.

Various molecular weights of the chitinase were reported on the microbial enzymes as valued from 40 to 200 kDa. The chitinase in our preparations had MW 54 kDa. Examination of the effect of metal ions showed that this enzyme was differently affected by these ions.

In this paper, the chitobiase produced by *Aeromonas* sp. J-5003 showed to be an alkaline and heat-labile enzyme. But since there has been few reports dealing the characteristics of microbial chitobiases, it was not practical compare our results with other microbial chitobiases. Recently, molecular clonings of chitobiase genes from several bacteria have been reported (Chitlaru and Roseman, 1996; Matsuo et al., 1999; Spindler-Barth et al., 1986; Tews et al., 1996), but it was also relatively less than those chitinases. So, it is necessary to study the cloning and analysis of the chitobiase gene from *Aeromonas* sp. J-5003.

## References

- Bassler, B.L., C. Yu, Y.C. Lee and S. Roseman. 1991. Chitin utilization by marine bacteria: Degradation and catabolism of chitin oligosaccharides by *Vibrio furnissii*. *J. Biol. Chem.*, 266, 24276–24286.
- Bhushan, B. and G.S. Hoondai. 1998. Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. *Biotech. Lett.*, 20, 157–159.
- Borooh, J., D.H. Leaback and P.G. Walker. 1961. Substrates for glucosaminidase. *J. Biochem.*, 78, 106–110.
- Chitlaru, E. and S. Roseman. 1996. Molecular cloning and characterization of a novel  $\beta$ -N-acetyl-D-glucosaminidase from *Vibrio furnissii*. *J. Biol. Chem.*, 271, 33433–33439.
- Choi, Y.U., J.H. Kang, M.S. Lee and W.J. Lee. 2003. Isolation and characterization of chitinase and chitobiase produced by *Aeromonas* sp. J-5003. *J. Kor. Fish. Soc.*, 6, 1–6 (in Korean).
- Fenton, D.M. and D.E. Eveleigh. 1981. Purification and mode of action of a chitosanase from *Penicillium islandicum*. *J. Gen. Microbiol.*, 126, 151–165.
- Hayashi, K., S. Sato, R. Takano, H. Tsujibo, H. Orikoshi, C. Imada, Y. Okami, Y. Inamori and S. Hara. 1995. Identification of the positions of disulfide bonds of chitinase from a marine bacterium, *Alteromonas* sp. strain O-7. *Biosci. Biotech. Biochem.*, 59, 1981–1982.
- Huang, J.H., C.J. Chen and Y.C. Su. 1996. Production of chitinolytic enzymes from a novel species of *Aeromonas*. *J. Ind. Microbiol.*, 17, 89–95.
- Jeuniaux, C. 1961. Chitinase, an addition to the list of hydrolases in the digestive tract of vertebrates. *Nature*, 192, 135–136.
- Jeuniaux, C. 1996. Chitinases. In: *Methods in Enzymology*, Vol. 8, E.R. Neufeld and V. Ginsberg, eds. Academic Press, New York, pp. 644–650.
- Kang, S.W. and M.J. Chung. 1999. Purification of chitinase from *Pseudomonas vesicularis* KW-15. *J. Chitin Chitosan*, 4, 132–136. (in Korean)
- Kim, S.K. 1997. Chitin and Chitosan; Base and Pharmacodynamics. Lee-Hwa Pub. Co. Seoul, Korea (in Korean).
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.
- Lee, J.T., D.H. Kim, J.H. Do and S.D. Kim. 1998. Purification and characterization of chitinase from antagonistic bacteria *Pseudomonas* sp. 3098. *Kor. J. Appl. Microbiol. Biotechnol.*, 26, 515–522. (in Korean)
- Lowry, O.H., N. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193, 265–275.
- Lundblad, G., M. Elander, J. Lind and K. Slettongren. 1979. Bovine serum chitinase. *Eur. J. Biochem.*, 100, 455–460.
- Matsuo, Y., M. Kurita, J.K. Park, K. Tanaka, T. Nakagawa, M. Kawamukai and H. Matsuda. 1999. Purification, characterization and gene analysis of N-acetylglucosaminidase from *Enterobacter* sp. G-1. *Biosci. Biotechnol.*

- Biochem., 63, 1261–1268.
- Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31, 426–428.
- Mitsuhiro, R. and M. Arai. 1992. Purification and some properties of chitinases from *Aeromonas* sp. No. 10S-24. *Biosci. Biotech. Biochem.*, 56, 460–464.
- Mitsutomi, M., T. Hata and T. Kuwahara. 1995. Purification and characterization of novel chitinases from *Stereomyces griseus* HUT 6037. *J. Ferment. Bioeng.*, 80, 153–158.
- Molano, J., I. Polacheck, A. Duran and E. Cabib. 1979. An endochitinase from wheat germ. Activity on nascent and preformed chitin. *J. Biol. Chem.*, 254, 4901–4907.
- Molloy, C. and B. Burke. 1997. Expression and secretion of *Janthinobacterium lividum* chitinase in *Saccharomyces cerevisiae*. *Biotech. Lett.*, 19, 1161–1164.
- Monreal, J. and E.T. Reese. 1969. The chitinase of *Serratia marcescens*. *Can. J. Microbiol.*, 15, 689–696.
- Ohtakara, A., Y. Uchido and M. Mitsutomi. 1978. Proceedings of the First International Conference on Chitin/Chitosan (R.A.A. Muzarell; E.R. Pariser, eds) MIT Sea Grant Report MITSG. 78, 587–606.
- Pelletier, A. and J. Sygusch. 1990. Purification and characterization of three chitosanase activities from *Bacillus megaterium* P1. *Appl. Environ. Microbiol.*, 56, 844–848.
- Sakai, K., A. Yokota, H. Kurokawa, M. Wakayama and M. Moriguchi. 1998. Purification and characterization of three thermostable endochitinases of a noble *Bacillus* strain, MH-1, isolated from chitin-containing compost. *Appl. Environ. Microbiol.*, 64, 3397–3402.
- Si, S.C. and Y.T. Li. 1970. Studies on the glycosidases of Jack bean meal. *J. Biol. Chem.*, 245, 5153–5160.
- Spindler-Barth, M., E. Shaaya and D. Spindler. 1986. The level of chitinolytic enzymes and ecdysteroids during larval-pupal development in *Epbestia cautella* and their modifications by a juvenile hormone analogue. *Insect Biochem.*, 16, 187–190.
- Tews, I., R. Vincentelli and C.E. Vorgias. 1996. N-acetylglucosaminidase (chitobiase) from *Serratia marcescens*; Gene sequence, and protein production and purification in *Escherichia coli*. *Gene*, 170, 63–67.
- Tsujibo, H., Y. Yoshida, C. Imada, Y. Okami, K. Miyamoto and Y. Inamori. 1991. Isolation and characterization of a chitin degrading marine bacterium belonging to the genus *Alteromonas*. *Nippon Suisan Gakkaishi*, 57, 2127–2131.
- Wadworth, S.A. and J.P. Zikakis. 1984. Chitinase from soybean seeds; Purification and some properties of the enzyme system. *J. Agric. Food Chem.*, 32, 1284–1288.
- Yabuki, M., K. Mizushima, T. Amatatsu, A. Ando, T. Fujii, M. Shimada and M. Yamashita. 1986. Purification and characterization of chitinase and chitobiase produced by *Aeromonas hydrophila* subsp. *anaerogenes* A52. *J. Gen. Appl. Microbiol.*, 32, 25–38.