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Effective Production of Chitinase and Chitosanase by *Streptomyces griseus* HUT 6037 Using Colloidal Chitin and Various Degrees of Deacetylation of Chitosan

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The advantages of the organism *Streptomyces griseus* HUT 6037 is that the chitinase and chitosanase using chitinaceouse substrate are capable of hydrolyzing both amorphous and crystalline chitin and chitosan. We attempted to investigate the optimization of induction protocol for high-level production and secretion of chitosanase and the influence of chitin and partially deacetylated chitosan sources (75~99% deactylation). The maximum specific activity of chitinase has been found at 5 days cultivation with the 48 hours induction time using colloidal chitin as a carbon source. To investigate characteristic of chitosan activity according to substrate, we used chitosan with various degree of deacetylation as a carbon source and found that this strain accumulates chitosanase in the culture medium using chitosanaceous substrates rather than chitinaceous substrates. The highest chitosanase activity was also presented on 4 days with 99% deacetylated chitosan.
The partially 53% deacetylated chitosan can secrete both chitinase and chitosanase which was defined as a soluble chitosan. The specific activities of chitinase and chitosanase were 0.89 at 3 days and 1.33 U/mg protein at 5 days, respectively. It indicate that chitosanase obtained from *S. griseus* HUT 6037 can hydrolyze GlcNAc-GlcN and GlcN-GlcN linkages by exo-splitting manner. This activity increased with increasing degree of deacetylation of chitosan. It is the first attempt to investigate the effects of chitosanase on various degrees of deacetylations of chitosan by *S. griseus* HUT 6037. The highest specific activity of chitosanase was obtained with 99% deacetylated chitosan.

Key words: *Streptomyces griseus* HUT 6037, chitinase/chitosanase, reducing sugar, soluble chitosan, deacetylated chitosan, induction time.

INTRODUCTION

Chitin, chitosan and its oligosaccharides, which are used very efficiency in the treatment of ulcerative colitis and other gastrointestinal inflammation disorders, can not be absorbed or digested directly in gastrointestinal tract but absorbed with only hydrolyzed oligosaccharide forms such as cellulose [1-3]. To produce these oligosaccharides, chemical procedure was conventionally used but caused environmental problems, and more of it was unsuitable for stability of human body and large-scale production of oligosaccharides. Therefore, studies on oligosaccharide production by microorganisms have been proceeding recently. Chitinase (poly-β-1→4-(2-acetamido-2-deoxy)-D-glucoside glycanhydrolase, [E.C. 3.2.1.14]) is defined as an enzyme which produces low-molecular-weight, soluble multimers of *N*-Acetyl-β-D-glucosamine (GlcNAc), the dimer *N,N'*-diacetyl chitobiose, while chitobiase (β-D-*N*-acetyl-glucosaminidase, [E.C. 3.2.1.30]) is defined as an enzyme which hydrolyzes the dimmer (chitobiose) to produce monomer (GlcNAc). Interaction between chitinase and chitobiase is needed for splitting chitin to GlcNAc [2,4-7]. They are present in a wide range of organisms including bacteria, insects, viruses, plants and animals and play important physiological and ecological roles [8]. Microorganisms such as *Serratia marcescens* [9-12], *Trichoderma harzianum* [13], *Streptomyces* sp. [14,15] and *Myrothecium verrucaria* [16] etc. produce extra-cellular chitinase/chitobiase. Chitinase/chitobiase are interested recently because of their possible application for the exploitation using of nature chitinaceous material and also for biochemical control of microorganism containing chitin. The chitosanase (E.C. 3.2.1.99) is identified as an enzyme that hydrolyzes-1,4-linkages between GlcNAc and D-glucosamine (GlcN) residues in a partially *N*-acetylated chitosan by an endowise manner. This enzyme has been obtained from microbial sources such as *Rhizopus rhizopodiformis*, *Bacillus* sp., *Bacillus* R-4, *Myxobacter* and *Streptomyces griseus* etc. [17,18]. The strain of *Streptomyces griseus*, the most active strain, can hydrolyze colloidal chitosan, soluble chitosan, glycol chitosan, carboxymethyl chitosan and carboxymethyl cellulose. It produces glucosamine oligosaccharide $(GlcN)_n$ (n=1~6) especially [19].

One of the advantages of using the organism *S. griseus* HUT 6037 is that the chitinase and chitosanase depending on chitinaceouse substrate are capable of hydrolyzing both amorphous and crystalline chitin and chitosan. Objectives in this study include selection of optimal substrate of microbial host (*Streptomyces*

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griseus HUT 6037) for chitinase/chitobiase and chitosanase production and secretion. We attempted to investigate the optimization of induction protocol for high-level production and secretion of chitosanase and the influence of chitin (powdered chitin, colloidal chitin) and variously deacetylated chitosan sources (75~99% degree of deactylation) on chitinase/ chitobiase and chitosanase production from *S. griseus* HUT 6037.

MATERIALS AND METHODS

Powdered Chitin

The purified chitin was purchased from Sigma Chemical Co. and ball-milled to 180~250 μm powder which was optimal partical size for the highest level of chitinase activity.

Colloidal Chitin

Various forms of colloidal chitin also were prepared according to the method of Berger and Reynolds [20]. The colloidal chitin solution was dialyzed against the 100 mM potassium phosphate buffer until a pH of 5 to 6 was maintained.

Soluble Chitosan

The powdered chitin (<210 μm) was hydrolyzed with 47% (w/w) sodium hydroxide solution at 25℃ for 56 hr and then neutralized with deionized water (about 80℃).

Deacetylated Chitosan with Degree of Deacetylation of 75~99%

The powdered chitin (100 g) was treated with 870 mL of 47% (w/w) sodium hydroxide solution at 110~120℃ for 1~3 hr under a nitrogen atmosphere. The chitosan was washed in water at about 80℃ to neutrality and the alkali treatment and washing in water were repeated two or more times to obtain chitosan products with 85~95% degree of deacetylation. To obtain over 95% deacetylated chitosan, fibrous chitosan was prepared before reaction of deacetylation [21]. Degree of deacetylation was measured using a modified method of Sannan *et al.* [22] and Domszy *et al.* [23,24]

Microorganism

S. griseus HUT 6037 used in this study produces chitinase and chitosanase and was kindly provided by Prof. Masaru Mitsutomi of Saga University, Department of Applied Biotechnology. The strain was maintained on agar slant containing 1.0% manitol, 0.2% peptone, 0.1% meat extract, 0.1% yeast extract and 0.05% $MgSO_4 \cdot 7H_2O$. This strain was transferred to 50 mL of a seed culture medium of pH 7.0, consisting of 1.0% manitol, 0.2% peptone, 0.1% meat extract, 0.1% yeast extract and grown at 30℃ for 24, 36 and 48 hr respectively to determine the optimal time of enzyme production. One mL of the seed culture was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of an enzyme production medium of pH 7.0, consisting of 0.05% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$ and 0.2% powdered chitin, colloidal chitin, soluble chitosan (53%) and variously deacetylated chitosan (75%, 85%, 95%, 99.9%) as a carbone sources, respectively, and cultivation was performed at 30℃ on a circular shaker.

Chitosanase Activity Assay

Chitosanase activity was measured with glycol chitosan as a substrate [25]. The reaction mixture containing 0.5 mL of 2% glycol chitosan in 0.1 M phosphate buffer of pH 7.5 and 0.5 mL of enzyme solution was incubated at 37℃ for 10 minutes at which time the reaction was stopped by boiling for 4 minutes. This solution was cooled in ice water after adding 1 mL of acetyl acetone and 1 mL of distilled water, and boiling for 20 minutes. Five mL of ethanol and Enrich reagent (DMAB) were added and incubated at 65~70℃ for 10 minutes. The absorbance was measured at 540 nm using ELISA plate reader (Anthos htⅢ, Anthos labtec. Instruments, Austria). Aminosugar liberated was determined by the method of Rondle and Morgan [26], using glucosamine as a standard. One unit of chitosanase activity was defined as the amount of the enzyme which liberated 1 mol of aminosugar per hour at 37℃.

Chitinase Activity Assay

Chitinase activity was measured by the colorimetric method of Ressig *et al.* [27,28] with some modification to allow for multi-sample analysis. The absorbance was measured at 545 nm using ELISA plate reader. One unit of activity was defined as the amount of enzyme able to liberate 1 mg of NAG per hour.

Chitobiase Activity Assay

Chitobiase activity was determined by measuring the amount of p-nitrophenol released when an aliquot of the enzyme solution was incubated with an aqueous solution of p-nitrophenyl-*N*-acetyl-D-glucosamine (pNP-NAG) [29]. Fifty mL of the 5 mM pNP-NAG solution was mixed with 20 mL of the enzyme solution appropriately diluted in Tris (hydroxy methyl aminomethane)-malate buffer at pH 7.0. After a 10 minutes incubation period at 37℃, the reaction was stopped by the addition of 100 mL of 0.25 M Na_2CO_3. The liberated p-nitrophenol was measured at a 405 nm absorbance in an ELISA plate reader. One unit of chitobiase activity is equal to the amount of enzyme necessary to liberate 1 mmol of p-nitrophenol per minute.

Determination of Reducing Sugar

The amount of total reducing sugar was determined by the method of Imoto and Yagishita modified method of Schales [30]. The reaction mixture containing 1.4 mL of 0.2% glycol chitin in McIlvaine buffer of pH 6.0 and 0.1 mL of enzyme solution. The mixture was incubated for 10 minutes at 30℃. The reaction was stopped by boiling for 15 minutes after the addition of 2 mL of 0.5 M sodium carbonate containing 0.05% potassium ferricyanide and cooled in an ice water. The amount of reducing sugars liberated was measured at 405 nm with ELISA plate reader.

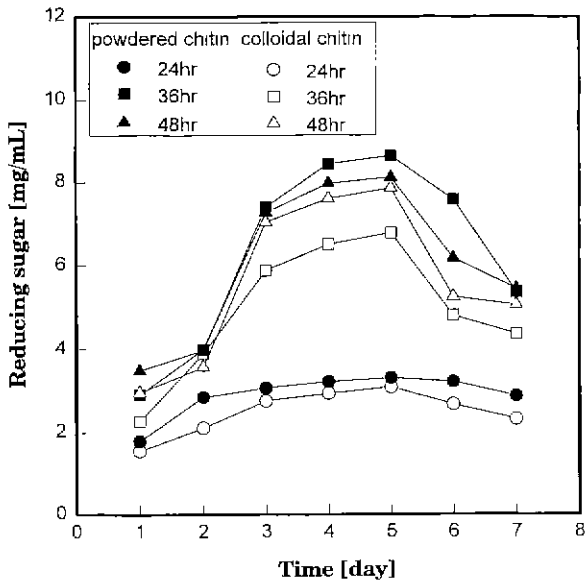

Fig. 1. Reducing sugar by *Streptomyces griseus* HUT 6037 for batch fermentation on powdered chitin and colloidal chitin at induction time (24, 36, 48 hr).

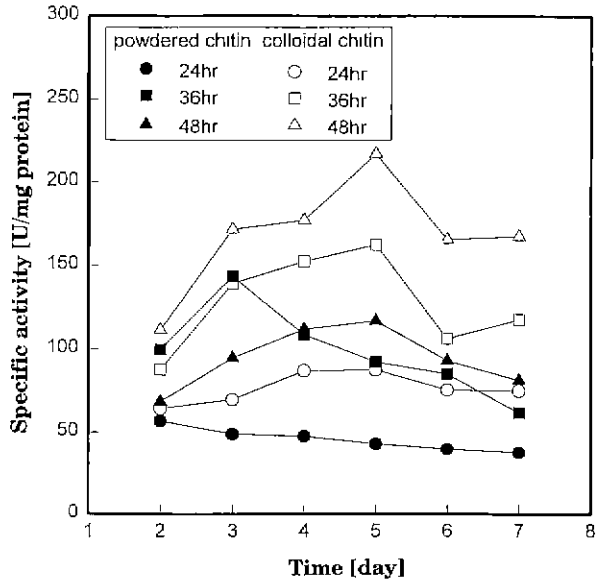

Fig. 2. Specific activity by *Streptomyces griseus* HUT 6037 for batch fermentation on powdered chitin and colloidal chitin at induction time (24, 36, 48 hr).

Total Protein Assay

Total protein concentrations were determined by the Bio-Rad protein assay, which follows the colorimetric procedure of Bradford [31]. For our studies, the assay was modified to accommodate the multisample format of an ELISA plate reader. All samples and standards were assayed in triplicate at an absorbance of 595 nm.

RESULTS AND DISCUSSION

Effect of Induction Protocol and Substrates on Chitinase

In the presence of powdered chitin and colloidal chitin as a sole carbon source, *Streptomyces griseus* HUT 6037 released chitinases in the batch fermentation. Induction protocol was varied 24, 36 and 48 hr according to one generation time. As shown in Fig. 1, the chitinase activity was higher on powdered chitin than on colloidal chitin and the highest activity was shown at 5 days cultivation where the induction time was 36 hr. It is of interest that *S. griseus* is capable of secreting different enzyme activity depending on a carbon source. The powdered chitin was more difficult to hydrolyze than colloidal chitin so that *S. griseus* might secrete chitinase of higher activity on powdered chitin. Each specific activity of chitinase according to cultivation time was presented in Fig. 2. The maximum specific activity of chitinase has been found at 5 days cultivation when the induction time was 48 hr using colloidal chitin as a carbon source. The specific activity of chitinase using colloidal chitin was higher than that of chitinase using powdered chitin. As a result, we found the optimization for producing chitinase by *S. griseus* which induction protocol and substrate used were 48 hr and colloidal chitin respectively through above experimental data.

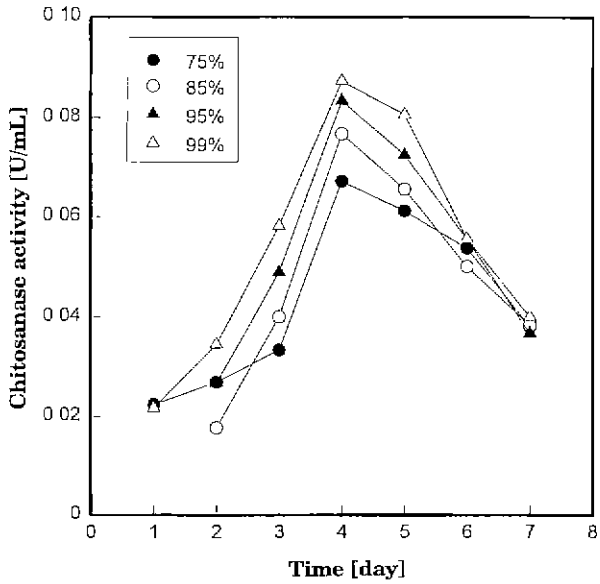

Fig. 3. Chitosanase activity by *Streptomyces griseus* HUT 6037 for batch fermentation using various degrees of deacetylation of powdered chitosan (75, 85, 95, 99%) at induction time (48 hr).

Effect of Deacetylated Chitosan on Enzymes

In a preliminary experiment, *S. griseus* produced chitinase on chitinaceous substrate sources. To investigate characteristic according to substrate, we used different deacetylated chitosan as a carbon source and found that this strain accumulates chitosanase in the culture medium using chitosanaceous substrates instead of chitinaceous substrates. As shown in Fig. 3, each chitosanase activities produced by various degrees of deacetylation of chitosan were the highest on 4 days cultivation in the enzyme production medium and especially, chitosanase activity of 99% deacetylated chitosan was higher than that of others. These results

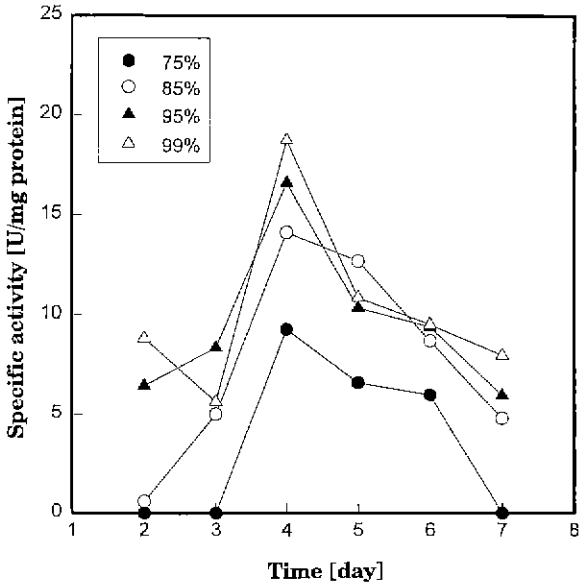

Fig. 4. Specific activity (chitosanase) by *Streptomyces griseus* HUT 6037 for batch fermentation using various degree of deacetylation of powdered chitosan (75, 85, 95, 99%) at induction time (48 hr).

represented that the activity of chitosanase increased with increasing degree of deacetylation of chitosan. Each specific activity of chitosanase depending on degree of deacetylation was presented in Fig. 4. The highest chitosanase activity was also presented on 4 days using 99% deacetylated chitosan. This result suggests that chitosanase from *S. griseus* HUT 6037 can hydrolyze GlcNAc-GlcN and GlcN-GlcN by exo-splitting manner so that the increase in degree of deacetylation of chitosan causes to increase chitosanase activity. Chitinase and chitobiase activities were also measured and the results were presented in Fig. 5 in which there was a little amount of chitinase and chitobiase, thus these enzymes could be ignored. This result was also identified the characteristic of enzyme produced by *S. griseus* HUT 6037. This strain was more suitable for producing chitosanase than chitinase.

Effect of Soluble Chitosan on Enzymes

We have previously found that enzyme of *S. griseus* HUT 6037 has activity on both chitinaceous substrate and 75~99% deacetylated chitosan. It is, therefore, assumed that 53% deacetylated chitosan, can secrete both chitinase and chitosanase. It is defined as a soluble chitosan. We attempt to investigate the mode of activity of enzyme using soluble chitosan as a carbon source. As mentioned in Fig. 3, the activity of enzyme from *S. griseus* using soluble chitosan differs from that of the enzyme using various degrees of deacetylation of chitosan. Chitinase, chitobiase and chitosanase activities are presented in Fig. 6. Chitosanase activity increased rapidly during 3 to 5 days of cultivation and then decreased at 7 days, whereas chitinase activity increased gradually until 5 days of cultivation and then decreased. Maximum activities of chitinase and chitosanase were 0.04 U/mL and 0.92 U/mL respectively at 5 days of cultivation. However chitobiase activity was rarely detected in this cultivation. Thus this results indicate that the enzyme cleaves

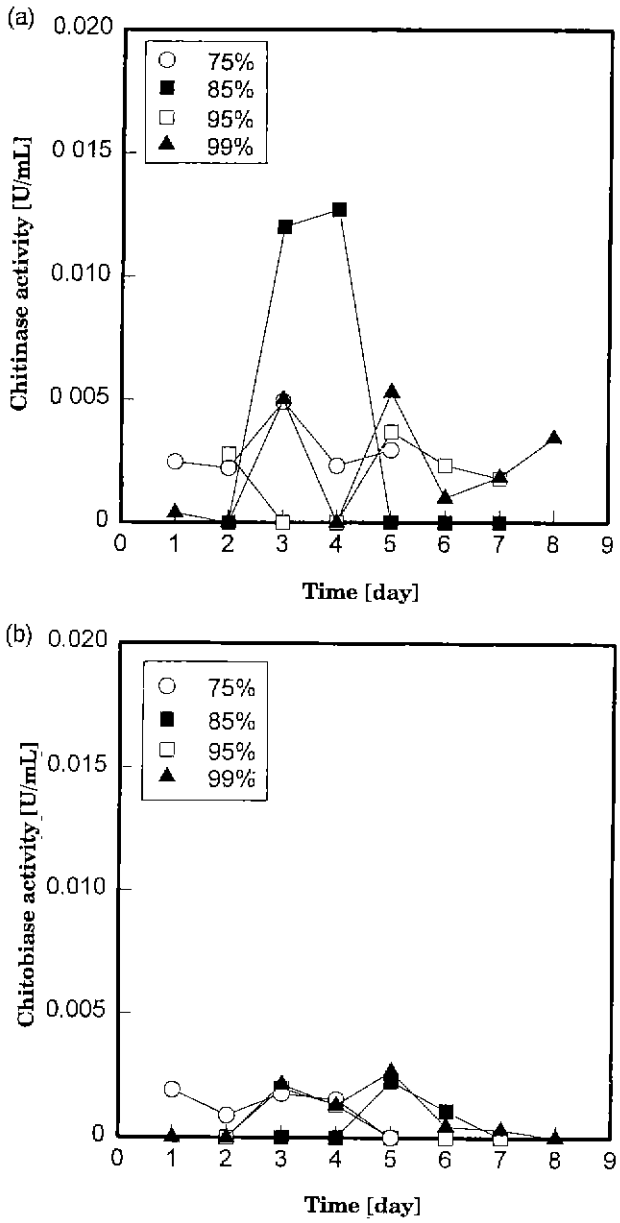

Fig. 5. Chitinase (a) and chitobiase (b) activity on *Sterptomyces griseus* HUT 6037 for batch fermentation using various degree of deacetylation of powdered chitosan (75, 85, 95, 99%) at induction time (48 hr).

both the *N*-acetyl-β-D-glucosaminidic and the β-glucosaminidic linkages in soluble chitosan molecules. We also showed specific activity of chitinase and chitobiase calculated by total amount of protein in Fig. 7. The specific activity of chitosanase was relatively high at 3 and 5 days of cultivation. The tendency of specific activity of chitinase was similar to that of chitosanase. The specific activities of chitinase and chitosanase were 0.89 at 3 days and 1.33 U/mg protein at 5 days, respectively. This result shows the optimum period for secretion of chitinase and chitosanase. Specific activities of chitinase and chitosanase produced by various substrates are summarized in Table 1 and we tried to compare with other experimental data.

In this study, we found that *S. griseus* HUT 6037 produced chitinase on chitinaceous substrate, chitosanase on chitosanaceous substrate and both enzyme on soluble chitosan (53% deacetylated chitosan). It is interest that *S. griseus* HUT 6037 is capable of pro-

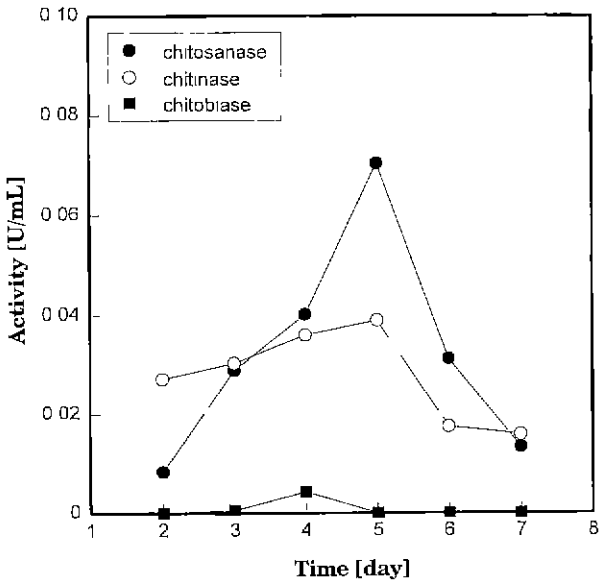

Fig. 6. Chitosanase, chitinase and chitobiase activity on soulble chitosan (degree of deacetylation 53%).

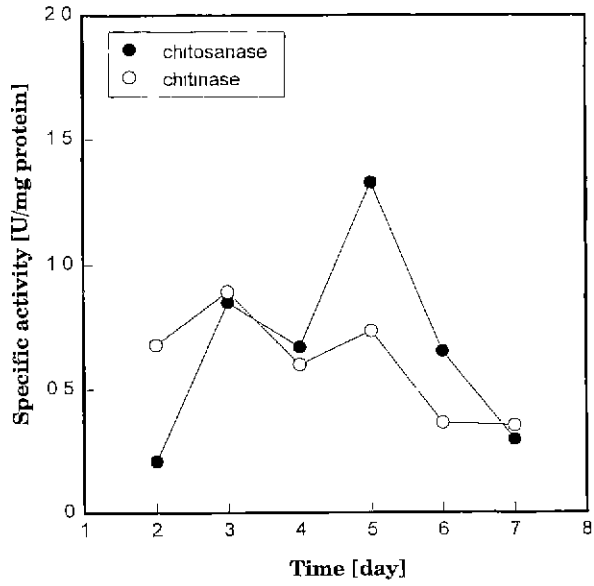

Fig. 7. Specific activity on *Streptomyces griseus* HUT 6037 for batch fermentation using soluble chitin at induction time (48 hr).

ducing either chitosanase or chitinase depending upon substrate used as a carbon source. The optimal induction protocol and substrate for producing the chitinase by *S. griseus* HUT 6037 was 48 hr and colloidal chitin as a carbon source in 5 days cultivation, respectively. The specific activity of this crude chitinase using a powdered chitin as a carbon source was about 2 times higher than that of purified chitinase by Mitsutomi *et al.* [7].

In addition, we also examined that specific activity of chitosanase was depending on degree of deacetylation. Specific activity of chitosanase using powdered chitosan with various degrees of deacetylation was much higher than using colloidal chitosan, glycol chitosan and CM-chitosan. Thus, these results clearly show that increase in the degree of deacetylation of chitosan was accor

Table 1. Comparison of specific activity of chitinase and chitosanase with various substrates

Substrate	Specific activity (U/mg protein)		Reference
	Chitinase	Chitosanase	
Colloidal chitin	12.56	–	This work
	16.00	–	[7]
Powdered chitin	8.29	–	This work
	4.10	–	[7]
Soluble chitosan	1.91	1.33	This work
	3.61	–	[7]
75% chitosan	–	15.18	This work
85% chitosan	–	16.60	This work
95% chitosan	–	12.69	This work
99% chitosan	–	18.73	This work
Colloidal chitosan	–	5.48	[7]
Glycol chitosan	–	3.59	[7]
CM-chitosan	–	5.44	[7]
CM-chitin	28.8	–	[7]

dance with increase in specific activity of chitosanase. It indicated that chitosanase obtained from *S. griseus* HUT 6037 could hydrolyze 1,4 linkage of GlcN-GlcN better than that of GlcNAc-GlcN and GlcNAc-GlcNAc in chitosan molecules according to increment of degree of deacetylated chitosan.

As mentioned above, this is the first investigated the effects of chitosanase on various degrees of deacetylation of chitosan by *S. griseus* HUT 6037. The highest specific activity of chitosanase was also presented 4 days cultivation using 99% deacetylated chitosan as a carbon source. We are now studying the purification and production of chitosan oligosaccharides from *S. griseus* HUT 6037. Furthermore, the component of chitosan oligosaccharide will be investigated by the analysis with high performance liquid chromatography (HPLC).

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REFERENCES

[1] Sakai, M. (1989) Development and present of chitin and chitosan oligosaccharides, *New Food Industry* 31(6): 17-25.
[2] Khoka, O. (1990) Chitinolytic enzymes. *Sen-I Gakkaishi* 46(12): 581-585.
[3] Takiguchi, Y. and K. Shimahara (1989) The production and utilization of *N*-acetylchitooligosaccharides and chitooligosaccharides by microorganism, *Bioindustry* 6(8): 616-621.
[4] Joshi, S., M. Kozlowski, S. Richens, and D. M. Comberbach (1989) Chitinase and chitobiase production during fermentation of genetically improved *Serratia liquefaciens*. *Enzyme Microb. Technol.* 11: 289-296.
[5] Mitsutomi, M., A. Ohtakara, T. Fukamizo, and S. Goto (1990) Action pattern of aeromonas hydro-

phila chitinase on partially *N*-acetylated chitosan. *Agric. Biol. Chem.* 54(4): 871-877.
[6] Mitsutomi, M., H. Kidoh, H. Tomita, and T. Watanabe (1995) The action of bacillus circulans WL-12 chitinase on partially *N*-acetylated chitosan. *Biosci. Biotechnol. Biochem.* 59(3): 529- 531.
[7] Mitsutomi M., T. Hata, and T. Kuwahara (1995) Purification and characterization of novel chitinases from *Streptomyces griseus* HUT 6037. *J. Ferment. Bioeng.* 80(2): 153-158.
[8] Ohno, T., S. Armand, T. Hata, N. Nikaidou, B. Henrissat, M. Mitsutomi, and T. Watanabe (1996) A modular family 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT 6037. *J. Bacteriol.* 178(17): 5065-5070.
[9] Monreal J. and E. T. Reese (1969) The chitinase of *Serratia marcescens*. *Can. J. Microbiol.* 15: 689.
[10] Reid, J. D. and D. M. Ogrydziak (1981) Chitinase-overproducing mutant of *Serratia marcescens*. *Appl. Environ. Microbiol.* 41(3): 664-669.
[11] Roberts, R. L. and E. Cabib (1982) *Serratia marcescens* chitinase: One-step purification and use for the determination of chitin. *Anal. Biochem.* 127: 402-412.
[12] Fuchs, R. L., S. A. McPherson, and D. J. Drahos (1986) Cloning of a *Serratia marscens* gene encoding chitinase. *Appl. Environ. Microbiol.* 51: 504-509.
[13] Ulhoa, C. J. and J. F. Peberdy (1992) Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. *Enzyme Microb. Technol.* 14: 236-240.
[14] Neugebauer, E., B. Gamache, C. V. D., and R. Brzezinski (1991) Chitinolytic properties of *Streptomyces lividans*. *Arch. Mircobiol.* 156: 192-197.
[15] Robbins, P. W., C. Albright, and B. Benfield (1988) Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. *J. Biol. Chem.* 263(1): 443-447.
[16] Vyas, P. and M. V. Deshpande (1989) Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. *J. Gen. Appl. Microbiol.* 35: 343-350.
[17] Zikakis, J. P. (1984) Chitin, Chitosan, and Related Enzymes. 1st ed., p. 161-179, Academic Press, Orlando.
[18] Muzzarelli, R. A. A. (1977) Chitin. 1st ed., p. 164-167, Pergamon Press, Oxford.
[19] Ohtakara, A., H. Ogata, Y. Taketomi, and M. Mitsutomi (1984) Purification and Charaterization of Chitosanase from *Streptomyces griseus*. p.147-160, Academic Press, Orlando.
[20] Berger, L. R. and D. M. Reynolds (1958) The chitinase system of a strain of *Streptomyces griseus*. *Biochim. Biophys. Acta.* 29: 522-534.
[21] Mima, S., M. Miya, R. Iwamoto, and S. Yoshikawa (1983) Highly deacetylation chitosan and its properties. *J. Appl. Polymer Sci.* 28: 1909-1917.
[22] Sannan, T., K. Kurita, and Y. Iwakura (1976) Effect of deacetylation on solubility. *Makromol. Chem.* 177: 3589-3600.
[23] Domszy, J. G. and G. A. F. Roberts (1985) Evaluation of infrared spectroscopic techniques for analyzing chitosan. *Makromol. Chem.* 186: 1671-1677.
[24] Sannan, T., K. Kurita, K. Ogura, and Y. Iwakura (1978) Studies on chitin: 7. IR spectroscopic determination of degree of deacetylation. *Polymer* 19: 458-459.
[25] Ohtakara, A. (1988) Chitosanase from *Streptomyces griseus*, Methods in Enzymology, 161, p. 64-69. Academic Press.
[26] C. J. M. Rondle and W. T. J. Morgan (1955) The determination of glucosamine and galactosamine. *J. Biochem.* 61: 586-589.
[27] Reissig, J. L., J. L. Strominger, and L. F. Leloir (1955) A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. Biol. Chem.* 27: 959-966.
[28] Sigma Chemical Co. (1994) Sigma quality control test procedure enzymatic assay of chitinase (EC 3.2.1.14), St. Louis, MO.
[29] Sigma Chemical Co. (1994) Sigma quality control test procedure enzymatic assay of β - N-acetylglucosaminidase(EC 3.2.1.30), Sigma Prod. No. A-3189. St. Louis, MO.
[30] Imoto, T. and Yagishita, K. (1971) A simple activity measurement of lysozyme. *Agric. Biol. Chem.* 35: 1154-1156.
[31] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248- 254.