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Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase

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Abstract—*Bacillus subtilis* W-118, a strain that produces antifungal materials, excreted a chitinase when cultured in a medium containing shrimp- and crab-shell powder as the major carbon source. This chitinase, purified by sequential chromatography, had a molecular mass of 20,600 Da and a p*I* of 6. The optimum pH, optimum temperature, and pH stability of the chitinase were pH 6, 37 °C, and pH 5–7, respectively. The unique characteristics of the purified chitinase include low molecular mass and acidic p*I*. In the investigation of the inhibitory activity, it was found that the growth of *Fusarium oxysporum* was 100% inhibited after incubation for 1 day with sterilized W-118 chitinase solution (5.6 units/mL). The chitinase hydrolyzates of chitin with low degrees of polymerization (DP 1–6) were analyzed by HPLC. Longer reaction times led to the generation of chitin oligosaccharides with lower DP. The chitin oligosaccharides were examined for their inhibitory effects on *F. oxysporum* and human leukemia cell lines.
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Keywords: Chitinase; *Bacillus subtilis*; Antifungal; Chitin oligosaccharides; Antitumor

1. Introduction

Chitin, a homopolymer of 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, GlcNAc) residues linked by β-(1→4) bonds, is a common constituent of insect exoskeletons, shells of crustaceans and fungal cell walls. The production of chitinases (EC 3.2.1.14) in higher plants may be part of their defense mechanism against infection by fungal pathogens. On the other hand, bacteria may produce chitinases for assimilation of chitin as carbon and nitrogen sources.[1,2] It is suggested that chitinolytic microorganisms or chitinolytic enzymes have potential applications in the biocontrol of plant pathogenic fungi and insects,[3] as a target for biopesticides,[4] and in many other biotechnological areas.[5] Chitooligosaccharides and their N-acetylated analogues are useful for applications in various fields because they have specific biological activities such as antimicrobial activity, antitumor activity, immuno-enhancing effects, and protective effects against infection with some pathogens in mice.[6–9]

Chitin oligosaccharides can be obtained by either chemical or enzymatic hydrolysis of chitin. In the case of chemical hydrolysis, chitin oligosaccharides were prepared by partial hydrolysis of chitin with concentrated HCl, phosphoric acid, or HF. However, acidic hydrolysis produced lower yields of oligosaccharides and a large amount of monomeric *N*-acetylglucosamine.[10–14] In addition, the oligosaccharides prepared by acidic hydrolysis might be toxic because of chemical changes during conversion.[10] Moreover, acid hydrolysis used for the chitin oligosaccharide production results in many problems (or defects), including an acid corrosion problem, difficulty in the control of reaction conditions, poor repeatability, and requirement of desalting due to the high concentration of salts formed in neutralization.[10] Therefore, enzymatic hydrolysis of chitin or chitosan is

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attracting growing interest. Recently, *Bacillus subtilis* IMR-NK1 chitosanases,[11] *B. subtilis* KH1 chitosanases,[12] *Aeromonas hydrophila* H-2330 chitinases,[14] and *Streptomyces kurssanovii* RCM-Ac-1504 D chitinases[15] have been studied for the preparation of chitosan oligosaccharides and chitin oligosaccharides. Besides, commercial enzymes such as cellulase,[13] pectinase,[16] papain,[17] and lysozyme[18] could also be investigated for the feasibility of production of oligomers by hydrolysis of chitin and chitosan.[13–18]

We have previously investigated the bioconversion of shrimp- and crab-shell powder (SCSP) of marine waste for bio-fungicide production. *B. subtilis* W-118, which was isolated from the soil in Taiwan, extracellularly produces antifungal materials.[19] Except for a chitosanase from *B. subtilis* KH1[11] and a chitosanase from a mutant of *B. subtilis* IMR-NK1,[12] specific features of the synthesis and secretion of chitinolytic enzymes by strains of *Bacillus* species have not been characterized in detail, unlike the case of actinomycetes and *Serratia marcescens*, which serve as sources of commercially available chitinases.[20] In the present work, we found *B. subtilis* W-118 displayed chitinolytic activity when cultured in a SCSP medium. To further study the relationship between the chitinolytic activity and antifungal activity, the purification and characterization of the novel chitinase, which has a low molecular mass and acidic p*I*, were investigated. Besides, the antitumor activities of the chitin hydrolyzates were also investigated.

2. Materials and methods

2.1. Materials

The shrimp- and crab-shell powder (SCSP) used in these experiments was prepared in our laboratory. In the preparation of the SCSP, the shrimp and crab shells collected from the marine food processing industry were washed thoroughly with tap water and then steamed. The solid material so obtained was dried, milled, and sieved to powder with diameters of <0.053 mm. DEAE-Sepharose CL-6B, Sephacryl S-200, and PBE 94 were from Pharmacia. Powdered chitin, hen egg-white lysozyme (HEWL), and Trypan Blue solution (0.4%) were purchased from Sigma Chemical Co., St. Louis, Mo. A standard mixture of *N*-acetylchitooligosaccharides, $(GlcNAc)_{1-6}$, was purchased from Seikagaku Kogyo Co., Japan. WST-1 (4-[3-(4-iodophenyl)-2-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate] was from Roche company (Germany). RPMI-1640 culture medium, FBS (fetal bovine serum), penicillin, streptomycin, and L-glutamine were from Gibco Company. Chitin powder was from Wako Chemicals (Japan). Microprotein assay kits were obtained from the Bio-Rad Company. Human chronic myelogenous leukemia (CML) cell line K562 was purchased from American Type Culture Collection (ATCC, Maryland, USA). Colloidal chitin was prepared from powdered chitin (Wako chemicals, Japan) by the method of Jeniaux.[21] All other reagents used were of the highest grade available.

2.2. Microorganism

B. subtilis W-118 was isolated from the soil in Taiwan and maintained on nutrient agar plates at 37 °C.[19] The plant-pathogenic fungus used in this study was *Fusarium oxysporum* CCRC35100 (from the Culture Collection and Research Center, Taiwan).

2.3. Chitinase production and purification

For the production of chitinase, *B. subtilis* W-118 was grown in 100 mL of medium (3% wt/vol shrimp- and crab-shell powder, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 50 mM sodium phosphate buffer, pH 6) in a 250-mL Erlenmeyer flask at 30 °C. For reflecting the growth of the culture in this medium by the OD_{660} measurement, the residual chitin was allowed to settle out.

Culture supernatant was collected from 3-day-old cultures by centrifugation at 12,000*g* for 20 min. The supernatant (505 mL) was concentrated by precipitation with $(NH_4)_2SO_4$ (608 g/L), the concentrated fraction was dialyzed with 10-kDa cut-off membrane against 50 mM sodium phosphate buffer (pH 6), and the enzymes were separated by DEAE-Sepharose column chromatography, followed by Sephacryl S-200 gel-filtration chromatography. The protein peak fractions containing chitinase activity were collected, concentrated with $(NH_4)_2SO_4$, and dialyzed against 50 mM sodium phosphate buffer (pH 6).

2.4. Measurement of enzyme activity

Chitinase activity was measured with colloidal chitin as a substrate. Enzyme solution (0.5 mL) was added to 1.0 mL of substrate solution, which contained a 1.5% suspension of colloidal chitin in a phosphate buffer (50 mM, pH 6), and the mixture was incubated at 37 °C for 15 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita[22] with *N*-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of the enzyme that produced 1 μmol of reducing sugar per minute.

Protein content was estimated by the method of Bradford,[23] using Bio-Rad protein dye reagent concentrate (Bio-Rad). Bovine serum albumin was used as the standard.

2.5. Determination of molecular mass and isoelectric point

The molecular mass of the purified chitinase was determined by the Agilent 2100 Bioanalyzer and Protein 200 Lab Chip kit (Agilent Technologies, Waldbronn, Germany). Gel–dye mix was first pipetted in the well on the protein chip and allowed to stand for 60 s, and the remaining solution was then removed. After the gel set, 12 μL of each gel–dye mix and destain solution were added into each well. Protein samples were denatured by mixing 4 μL of the protein sample and 2 μL of the denaturing solution, containing 4% SDS, 290 μg/mL myosin internal (upper) marker and 3% β-mercaptoethanol, and heating to 100 °C for 5 min. Following conventional protocol by centrifugation at 12,000*g* for 15 s, each sample and standard were added with 84 μL lower marker mix (Agilent Technologies No. 5056-4430, excitation/emission 650/680 nm) in deionized water and loaded on the protein chip in a volume of 6 μL. The chip was placed in the Agilent 2100 Bioanalyzer (Agilent Technologies, G2940AA) and started immediately. Both separation and detection were carried out in the Agilent 2100 Bioanalyzer instrument, which uses epifluorescence detection with a low semiconductor laser that emits at 630 nm. Images were recorded on a Nikon CCD camera (Hammamatsu C2400-60, Technical Instrument, San Francisco, CA, USA).[24] The isoelectric point of the purified chitinase was estimated by chromatofocusing. The chitinase solution (1 mL) was loaded onto a chromatofocusing PBE 94 column (1 by 40 cm) equilibrated with 25 mM Histine·HCl buffer (pH 6.2), and the elution was done with Polybufer 74·HCl (pH 4.0) as described in the manufacturer's manual (Pharmacia).

2.6. Measurement of antifungal activity of W-118 chitinase

The antifungal activity for the supernatant obtained above was estimated using a growth-inhibition assay described earlier.[25] To test the antifungal inhibitory effect of the enzyme obtained above, Petri plates were filled with 5 mL of molten PDA pre-cooled to 45 °C and divided into two groups (triplicate for each). To each plate in the experimental group (*E*), a properly diluted enzyme solution (5 mL) was added. The ratio (vol/vol) of the enzyme and PDA added in the Petri plates was 1:1. To those of the control group (*C*) was added an equal amount of sterile water instead of enzyme solution. After the plates were cooled, the fungal spores (20 μL) were then placed onto an agar surface. Both groups were incubated at 25 °C. The diameters of the largest and smallest fungal colonies were recorded, and the averages were calculated. The inhibition ratios were calculated with the following formula:

$$\text{Inhibition ratio } (\%) = (C - E)/C \times 100\%$$

where *C* is the average diameter of the largest and smallest colonies of the control groups and *E* is the average diameter of the largest and smallest colonies of the experimental groups.

2.7. Analysis of oligosaccharides by HPLC

For the preparation of oligosaccharides, colloidal chitin (3 g) was suspended in 100 mL of 50 mM of sodium phosphate buffer (pH 6). To the suspension, enzyme (20.5 mg) was added and shaken at 37 °C for 60 min. After being placed in a 100 °C water bath for 15 min to inactivate the enzyme, the insoluble materials from the enzymatic hydrolysis were removed by centrifugation (3000 rpm). The water-soluble fraction was concentrated to 20% of the original volume, followed by addition of MeOH (9 × vol). The suspension was centrifuged. The precipitate was collected and immediately vacuum-dried. This dried material was composed of oligosaccharides from chitin.

The compositions of the oligosaccharides obtained by enzymatic hydrolysis as described above were determined by HPLC (Hitachi L-7100 apparatus) on a Phenomenex LUNA NH_2 column (4.6 mm × 250 mm; Phenomenex, Torrance, CA, USA) at 50 °C. Then, a 20-μL of sample was chromatographed using linear gradients of CH_3CN–H_2O from 70% to 55% in 30 min at a flow rate of 1 mL/min. The oligosaccharides were monitored at 205 nm with a spectrophotometric detector.

2.8. Cell culture and chitooligomer mixture treatment

The human chronic myeloid leukemia (CML) cell line K562 was cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and maintained in an exponential growth state. The hydrolyzed chitooligomer mixtures were dissolved in PBS, sterilized, and stored at −20 °C until used. For assay of cytotoxicity, K562 cells were incubated in 35-mm Petri-dishes at an initial concentration of 1×10^5 cells/mL in the presence of chitooligomer mixtures.

2.9. Cell-proliferation assay

Chitooligomer mixture-treated K562 cells were collected by gently rubbing the dishes with a rubber policeman (Bellco Glass, Vineland, USA). The numbers of viable cells were counted on days 1, 2, and 3 using the Trypan Blue dye exclusion test[26] or a colorimetric method.[27] In brief, the chitooligosaccharide mixture and cells were put into 96-wells, followed by incubation in an incubator at 37 °C, 5% CO_2, 95% RH for 24, 48, 72 h. After incubation, 10 μL WST-1 reagent was added, followed by incubation in an incubator at 37 °C, 5% CO_2, 95% RH for 4 h. An orange-colored formazan formed. The absorbances at 450 and 630 nm were measured with an

ELISA reader. The inhibition rate by the cells killed was calculated as follows:

$$\text{Inhibition rate } (\%) = [1 - \mathrm{OD_C}/\mathrm{OD_T}] \times 100\%$$

where $\mathrm{OD_C}$ is the absorbance of the control group without test sample and $\mathrm{OD_T}$ is the absorbance of the experimental group with the various test samples.

2.10. Morphological changes of K562 cells

After 5 days of treatment, cells were collected and cytocentrifuged onto a microscope slide using Cytospin® (Shandon Southern Instrument, Inc.), stained with Wright's stain, and observed under an inverted microscope (Olympus) with a magnification of 1000×.

3. Results and discussion

3.1. Production and purification of W-118 chitinase

B. subtilis W-118 was grown aerobically in 100 mL of the optimum medium in a 250-mL Erlenmeyer flask at 30 °C. During the process of incubation, chitinase activity, cell growth, and pH of the broth were measured. The time courses of cell growth and chitinase activity are shown in Figure 1. The chitinase activity increased along with the cell growth (as judged by $\mathrm{OD_{660}}$) and reached maximum (4.2 unit/mL) when the cell growth reached mid-exponential phase at 3 days of incubation. In the media without SCSP, chitinolytic activity was not found in the fermentation broth (data not shown). Consequently, it was inferred that chitinolytic enzymes produced by *B. subtilis* W-118 should be induced enzymes.

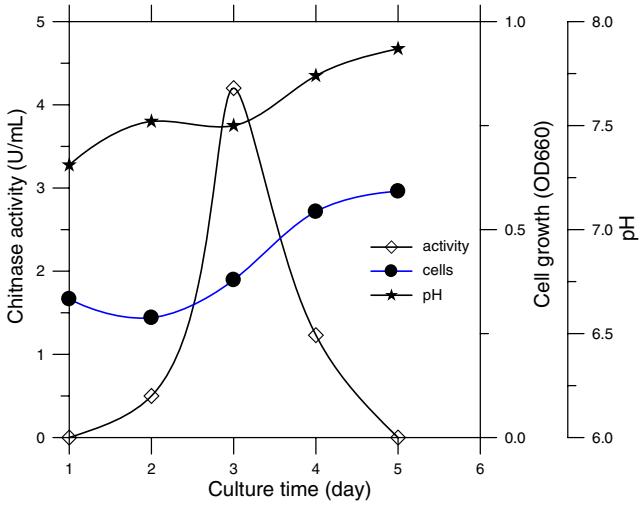

Figure 1. Time courses of growth (●), chitinase activity (◊), and pH (★) in a culture of *B. subtilis* W-118. OD, optical density.

When grown on SCSP as a major carbon source, *B. subtilis* W-118 released chitinases into the culture fluid with the highest activity at 3 days. The purification of the chitinase from the culture supernatant (505 mL) was described under Materials and Methods (Section 2.3). Upon ion-exchange chromatography on a DEAE-Sepharose CL-6B column, one protein peak exhibiting chitinase activity was resolved in the fractions eluted by a buffer containing 0–1.0 M NaCl (Fig. 2), allowing each to be pooled separately. The purification procedures are summarized in Table 1. The purification steps were very effective and combined to give overall purifications of sevenfold. The overall activity yield of the purified chitinase was 21%, with specific chitinase activity of 7.1 unit/mg of protein. The final amount of the purified chitinase obtained was 63 mg. The purified chitinase was also confirmed to be homogeneous by the Protein 200 Chip and chromatofocusing (data not shown).

Similar to chitinase-producing strains of *Bacillus cereus* strain 65[28] and *B. cereus* YQ308,[29] and chitosanase-producing strains of *B. subtilis* IMR-NK1,[11] we detected one chitinolytic enzyme in the culture supernatant of *B. subtilis* W-118. Although there have been four distinct chitinases purified from *B. cereus* CH, the difference in their molecular sizes may be caused by proteolytic modification of their carboxyl terminal regions.[30] A similar phenomenon was also found in the chitosanase- and protease-producing strain of *B. subtilis* IMR-NK1.[11] The recovery of the IMR-NK1 chitosanase activity was rather low at the final stage of purification. The authors suspected that some of the chitosanase activity loss occurred due to protease degradation before complete removal of protease activity during

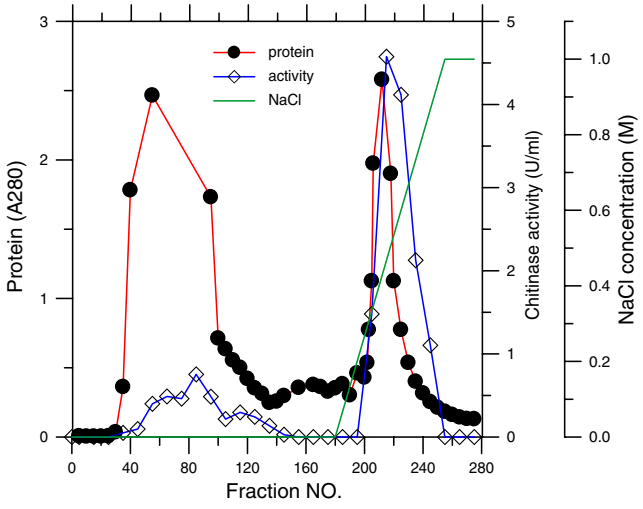

Figure 2. Elution profile of the chitinases on DEAE-Sepharose CL-6B. The DEAE-Sepharose CL-6B column (5 × 30 cm) pre-equilibrated with 50 mM sodium phosphate (pH 6) was eluted with a linear gradient of 0–1.0 M NaCl in 50 mM phosphate buffer at a flow rate of 75 mL/h.

Table 1. Purification of the chitinase from *B. subtilis* W-118

Step	Volume (mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Activity yield (%)
Culture supernatant	505	2071	2105	1.02	100
$(NH_4)_2SO_4$ precipitate	50	422	900	2.13	43
DEAE-Sepharose CL-6B	350	166	674	4.06	32
$(NH_4)_2SO_4$ precipitate	25	101	584	5.78	28
Sephacryl S-200	80	63	448	7.11	21

purification. In our preliminary experiment, we found the activity of W-118 chitinase was not affected by the protease partially purified from the culture supernatant of *B. subtilis* W-118 itself. From these results, it was speculated that the disappearance of the activity of chitinase or chitosanase might be directly related to the protease activity of the same strain.

Since shrimp- and crab-shell powder (SCSP) contains chitin and proteins, when it is used as carbon/nitrogen source, it induces the production of chitinase besides inducing the production of protease. This is different to other reports in which, to induce the production of chitinase, chitin or chitosan is used as the carbon/nitrogen source. It is interesting that chitin/chitosan, although more expensive than SCSP, was selected (because of tradition or experiment results) as the carbon/nitrogen sources by most other papers to induce chitinase/chitosanase production. This phenomenon might be relative to whether, besides chitinase/chitosanase, protease (which will degrade and thus inactivate the above enzymes) is present in the culture supernatant. Namely, when selecting chitin/chitosan as the inducers for the isolation of chitinase/chitosanase-producing strains, the chitinase/chitosanase secreted by the isolated strains would not be mis-estimated due to being degraded by protease. On the contrary, since shellfish chitin wastes are cheaper and can be easily recovered from the seafood industry, we used SCSP as the sole carbon/nitrogen source from the beginning of the screening of chitinase-producing strains. We found the chitinase produced by the isolated target strains seemed to be not inactivated by the protease simultaneously produced by the same strains. Because most insect eggs and shells contain both chitin and protein at the same time, these types of chitinase- and protease-producing microbial strains might be used as pesticides. The chitinase/protease-producing strains that can use shellfish chitin-containing wastes (such as SCSP) as the sole carbon/nitrogen source are especially more valuable to investigate as potential biocontrol agents.

3.2. Some properties of W-118 chitinase

The molecular mass of the chitinase was estimated by the Protein 200 Chip to be 20.6 kDa, and the p*I* of the chitinase was 6 by chromatofocusing. The molecular mass of W-118 chitinase is approximately 20,600 Da by the Protein 200 Chip, which is apparently far smaller than those known *Bacillus* chitinases (molecular mass ranging from 35,000 to 89,000 Da), except for two chitinases (14,400 and 16,900 Da) from *Bacillus amyloliquefaciens* V656.[25] Molecular masses are reported, for example, as follows: 48,000 Da for *B. cereus* YQ308;[29] 36,000 Da for *B. cereus* 6E1;[31] 36,000 Da for *B. cereus* 65;[28] 35,000, 47,000, 58,000, and 64,000 Da for *B. cereus* CH;[30] 68,000, 38,000, and 52,000 Da for *B. cereus* VKPM B-6838;[32] 74,000, 69,000, 38,000, 38,000, 39,000, and 52,000 Da for *Bacillus circulans* WL-12;[33,34] 45,000 Da for *B. circulans* No. 4.1;[35] 36,500 Da for *Bacillus* sp. NCTU2;[36] 41,000 Da for *Bacillus* sp. BG-11;[37] 35,000 and 46,000 Da for *Bacillus* sp. X-b;[38] and 71,000, 62,000, and 53,000 Da for *Bacillus* sp. MH-1.[39]

Most of the bacterial chitinases have acidic p*I*s, and actinomyces chitinases have neutral or alkaline p*I*s. Plant chitinases generally have very basic or very acidic isoelectric points. *B. subtilis* W-118 chitinase has a similar acidic p*I* (pH 6.0) as *B. cereus* chitinase (pH 6.4)[28] and *Bacillus* sp. NCTU2 chitinase (pH 6.3),[36] but it is different from the other reported *Bacillus* chitinases. The p*I*s are reported, for example, as follows: 5.2 for *B. cereus* YQ308;[29] 5.8 and 5.3 for *B. amyloliquefaciens* V656;[25] 4.5, 4.7, 5.2, 5.9, and 6.6 for *B. circulans* WL-12;[33] 5.1 for *B. circulans* No. 4.1,[35] and 5.3, 4.8, and 4.7 for *Bacillus* strain MH-1.[39]

Using colloidal chitin as a substrate under the standard assay conditions, maximum activity of the purified chitinase was at pH 6. It was stable at pH 5–7 for 30 min. The optimum temperature for chitinase activity was 37 °C over 30 min of incubation. It lost most of its activity at 60 °C over 30 min and was completely inactivated when incubated at 80 °C for 30 min. Similar to W-118 chitinase, some *Bacillus* chitinases also worked better at an acidic or near-neutral pH. Some optimum pH values are as follows: *B. cereus* YQ308 is at pH 7.0,[29] *Bacillus* sp. BG-11 is at pH 8.5,[37] *Bacillus thuringiensis* var. *caucasicus* INMI Arm.837 is at pH 8.0,[40] *B. circulans* No. 4.1 is at pH 8.0,[35] *B. cereus* 6E1 is at pH 5.8,[31] and *B. cereus* CH is at pH 5.0–7.5.[30] The low thermal stability of W-118 chitinase is similar to those observed for chitinases of other origins.[35,41–45]

The inhibitory activity of W-118 chitinase on growth of *F. oxysporum* was found to be 100% after 1 day of incubation with sterilized chitinase solution (5.6 U/mL).

Thereafter, inhibitory activity gradually decreased with the increase of incubation time. The inhibitory activity for three days of incubation decreased to only 10% of that of the 1 day of incubation (data not shown). The reason that the antifungal activity of W-118 chitinase decreased with incubation time was attributed to the fact that this enzyme is thermally sensitive. Besides, for the antifungal chitinases reported in published papers, all the hydrolysis mechanisms were of the *endo* type.[29] Therefore, the unsustainable antifungal activity of W-118 chitinase might also be attributed to its belonging to the *exo* type. To investigate this, the oligosaccharides from the hydrolysis of chitin by W-118 chitinase were analyzed with HPLC as follows.

3.3. Enzymatic hydrolyzates of colloidal chitin

The analysis of *N*-acetylchitooligosaccharides was found to be influenced by the ratio of acetonitrile–H_2O, giving results that were less accurate with an increase of the peak retention time. Therefore, the retention time of *N*-acetylchitooligosaccharides was studied. As shown in Figure 3, the natural logarithm of the retention time of *N*-acetylchitooligosaccharides in HPLC has a linear correlation with its degree of polymerization (DP). Therefore, a gradient elution was used, and the best separation was found to be when the ratio of acetonitrile–H_2O was lowered from 70:30 to 55:45 in 30 min. All $(GlcNAc)_{1-6}$ (DP 1–6; chitin oligosaccharides) could be eluted in 30 min, and the analysis could be improved from the decrease of the peak retention time. To clarify the cleavage, 3% of colloidal chitin was hydrolyzed at 37 °C by W-118 chitinase for 30, 60, 90, and 120 min, and the reaction products for each time period were analyzed by using HPLC. As shown in Figure 4, $(GlcNAc)_{1-6}$ could be detected from the sample after 30 min of hydrolysis. Thereafter, GlcNAc (monosaccharide) slowly increased with the reaction time; however, the relative ratio between $(GlcNAc)_{2-6}$ was not obviously changed.

The main function of bacterial extracellular hydrolases is, undoubtedly, the release of nutrients from different substrates for the needs of a bacterium. In addition, excreted enzymes alone, or with other compounds like antibiotics, may be used by bacteria for competition with other microbial species. Numerous microorganisms with antifungal activities have been identified, and many have been effective in field experiments. So far bacteria, especially *Pseudomonas* strains[46–50] and *Bacillus* strains,[20,38,51–53] have been intensively investigated as biological control agents. Roberts and Selitrennikoff[54,55] studied plant and bacterial chitinases for antifungal activity and enzyme specificity. According to their results, plant chitinases isolated from the grain of wheat, barley, and maize functioned as endochitinases and inhibited hyphal elongation of test fungi. In contrast, bacterial chitinases from *S. marcescens*, *Streptomyces griseus*, and *Pseudomonas stutzeri* act as exoenzymese and had no effect on hyphal extension of test fungi like *Trichoderma reesei* and *Phycomyces blackesleeanus*. Pleban et al.[28] found that the crude extracellular chitinase of an endophytic bacterium *B. cereus* 65 decreased spore germination of *F. oxysporum*. Ordentlich et al.[56] found crude chitinase of *S. marcescens* caused lysis of

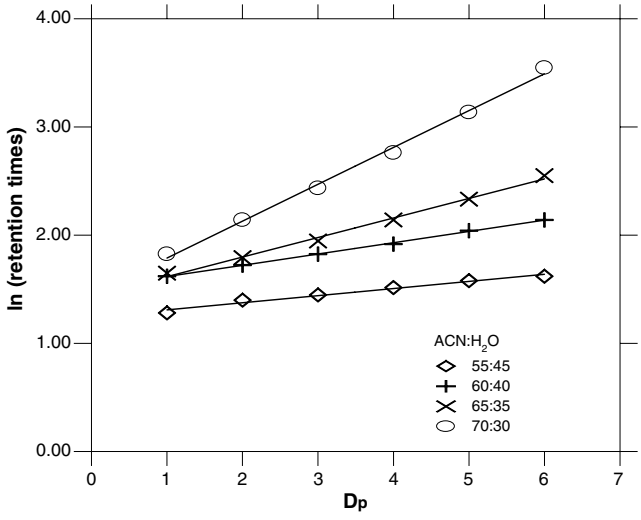

Figure 3. Semi-logarithmic relationship between the degrees of polymerization (DP) of *N*-acetyloligosaccharides and their retention times (in minutes) by HPLC. Mobile phase: acetonitrile–water (v/v) = ◇, 55:45; +, 60:40; ×, 65:45; and ○, 70:30.

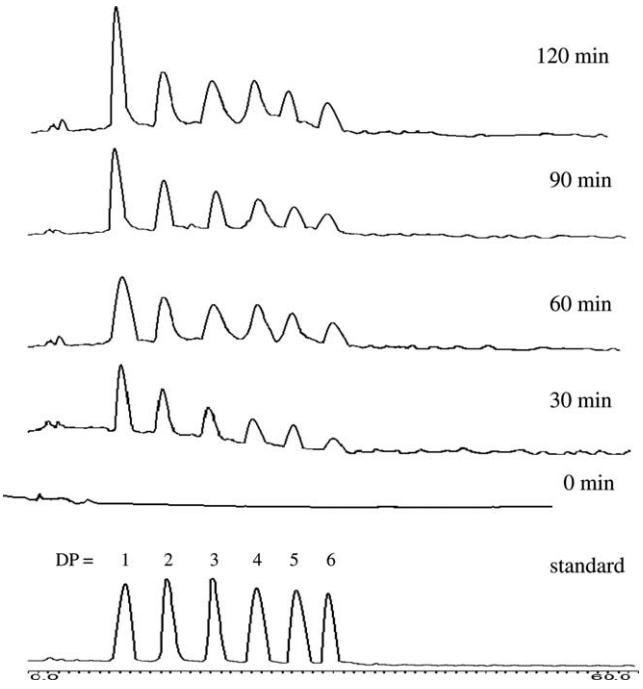

Figure 4. HPLC analysis of the reaction products from colloidal chitin after hydrolysis with W-118 chitinase.

hyphal tips of *Sclerotium rolfsii*. Although such bacterial chitinases are reported as possessing antifungal activities, the chitinase preparations that are used are the crude or partially purified chitinases. We have observed that *B. subtilis* W-118 chitinase inhibited the growth of the plant-pathogenic fungus, *F. oxysporum*. Similar phenomena were seen when plant-pathogenic fungi were treated with purified bacterial endochitinase from *B. cereus* YQ308,[29] and purified fungal endochitinase from *Trichoderma harzianum*[57,58] and *Gliocladium virens*.[59] From the results in Figure 4, the chitin hydrolyzates of W-118 exochitinase showed that GlcNAc (the monosaccharide) increased over time. This phenomenon was different to the other reported antifungal endochitinases. These results showed W-118 chitinase was more typical of an exochitinase. The reason why the antifungal activity of W-118 chitinase decreased with incubation time might be attributed to its belonging to *exo* type and to its thermal sensitivity.

Chitin oligosaccharides and chitosan oligosaccharides have recently attracted much attention for their various physiological activities, including antifungal activity, antitumor activity, and immuno-enhancing effect.[6–9] In particular, those with oligomers with a degree of polymerization equal to or greater than six have a strong biological activity. The antifungal and antiproliferative effects of chitin oligosaccharides from the hydrolysis of colloid chitin by W-118 chitinase were investigated as follows.

3.4. Antifungal and antiproliferative effects of chitin oligosaccharides

To investigate the effect of chitin oligosaccharides on the growth of *F. oxysporum* mycelia, the change in the appearance of the tested fungus following co-incubation with the oligosaccharides after 3 days in PDB medium was studied. Namely, to test the antifungal effect of the oligosaccharides obtained above, 10 μL of the fungal spores of *F. oxysporum* were added to an Eppendorf tube filled with 0.5 mL of potato/dextrose/broth (PDB) and 0.5 mL of the oligosaccharide mixture (experimental group). To those of the control group, an equal amount of sterile water instead of test mixture was added. As shown in Figure 5, a black color was found with partial sites of the *F. oxysporum* mycelia. Such an abnormal phenomenon was not found in the control group. So far, for the assay of antifungal activity, chitin, chitosan and their derivatives were most used as studied materials. For example, chitosan reduces the in vitro growth of numerous fungi with the exception of Zygomycetes, that is, the fungi containing chitosan as a major component of its cell wall.[60] The antifungal effect of chitosan on in vitro growth of fungal pathogens was studied by El Ghaouth et al.[61] These authors further confirmed the importance of a large number of alternating positively charged groups along the length of the polymer chain.[61,62] Besides, the oligosaccharides of chitin and chitosan have been reported to have antibacterial activity. For example, Tsai et al.[63] hydrolyzed chitosan with a commercial cellulase preparation, and tested the derived oligosaccharide mixture (DP 1–8). They found very strong antibacterial activity on bacteria such as *Aeromonas hydrophila*, *Escherichia coli*, and *Staphylococcus aureus*.

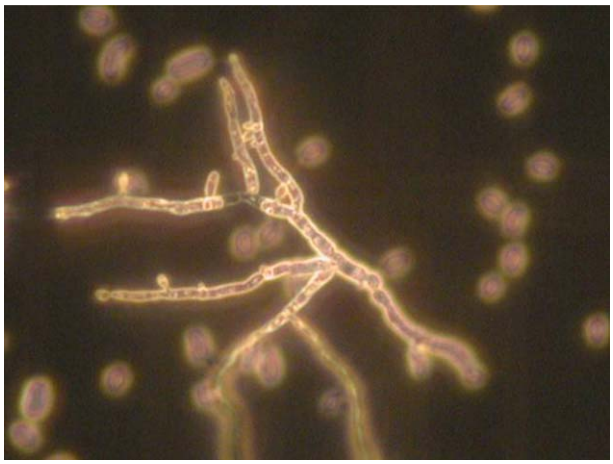

Figure 5. Mycelium and spores of *Fusarium oxysporum* grown in potato dextrose broth in the presence of the oligosaccharides mixture. Light microscopy at a magnification of 400×.

These research results showed that adding chitin oligosaccharides did not cause apparent changes such as the cleavage or swelling of the *F. oxysporum* mycelia,[19] but did make part of the *F. oxysporum* mycelia darken (Fig. 5). Since the culture supernatant of *B. subtilis* W-118 fermented SCSP contained W-118 chitinase besides antifungal compounds,[19] the darkening effect of chitin oligosaccharides on *F. oxysporum* mycelia might enhance the antifungal activity of the antifungal materials and chitinase produced by the strain W-118. For instance, chitin synthetase in *F. oxysporum* cells was inhibited by the chitin oligosaccharides or other unknown factors. This phenomenon has never been reported before and needs further detailed investigation to figure out the exact reason.

Pae and Seo[64] indicated that the water-soluble *N*-acetylchitooligosaccharides were capable of antitumor and apoptosis-inducing effects on human acute myeloid leukemia (AML) cells HL-60. In this study, the antiproliferative effects of chitin oligosaccharides on CML K562 cells were also selected for further investigation. Currently, the important index for effective anticancer drug screening is inhibiting the growth of the cancer cells by cell cycle arrest, apoptosis induction, and cell differentiation. WST-1 was used to detect the growth of K562 cells with the stimulation of high concentration of 1000 μg/mL of *N*-acetylchitooligosaccharides for 24, 48, and 72 h. As shown in Figure 6, the antiproliferative

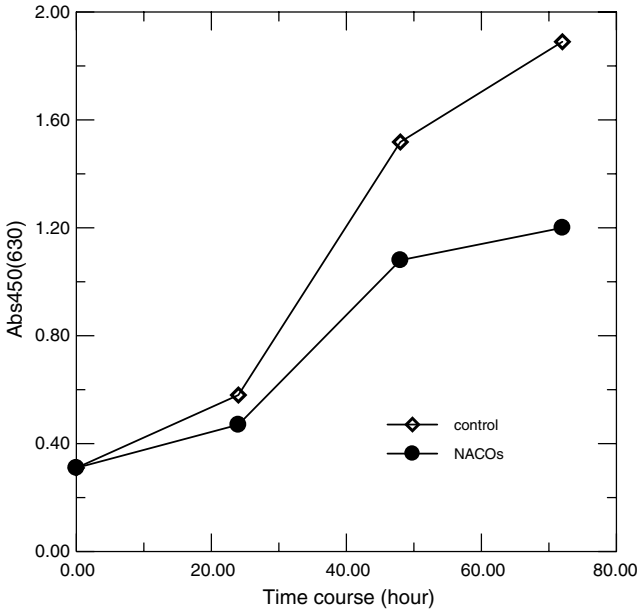

Figure 6. Effects of *N*-acetylchitooligosaccharides on anti-proliferation of K562 cells by WST-1 plot.

effects of the chitin oligosaccharides did not seem obvious. Even after treatment with high doses of chitin oligosaccharides (1000 μg/mL) for 24, 48, and 72 h, the growth rates of K562 cells were still increased to 29%, 36%, and 51%, respectively. After treatment with *N*-acetylchitooligosaccharides for 24, 48, and 72 h, the cell morphology was examined by phase-contrast microscopy. It was revealed that chitin oligosaccharides not only decreased the number of K562 cells, but the cells became wrinkled and showed shrunken forms (data not shown).

In summary, the characteristics of W-118 chitinase differ from the known *Bacillus* chitinases/chitosanases in terms of their molecular weights and isoelectric points. Compared to other known bacterial chitinases/chitosanases, the unique characteristics of W-118 chitinase include both a lower molecular weight and the first report of an antifungal exochitinase purified from *B. subtilis*.

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