

Chitinase Inhibitor Allosamidin Is a Signal Molecule for Chitinase Production in Its Producing *Streptomyces*

I. Analysis of the Chitinase Whose Production Is Promoted by Allosamidin and Growth Accelerating Activity of Allosamidin

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Abstract Allosamidin, a typical secondary metabolite of *Streptomyces*, has been known as a chitinase inhibitor. We found that allosamidin can dramatically promote chitinase production and growth of its producer, *Streptomyces* sp. AJ9463, in a chitin medium at a few hundred nM. Allosamidin promoted production of the main chitinase detected in the culture filtrate and the chitin-hydrolytic activity of the chitinase was not inhibited by allosamidin at the concentration. The gene encoding the chitinase showed that it is a family 18 chitinase and it was revealed that two genes encoding proteins constructing two-component regulatory system were present at 5'-upstream region of the chitinase gene. Allosamidin is located in the microbial mycelia cultured in a medium without chitin, but it was released from the mycelia by responding to chitin. These results show that allosamidin acts as a key signal molecule for chitinase production in its producing strain, which may be useful for its growth in chitin-rich environment.

Keywords allosamidin, chitinase, *Streptomyces*, two-component regulatory system, signal molecule

Introduction

Allosamidin is a typical secondary metabolite of *Streptomyces* without antibiotic activity [1]. It has a unique pseudotrisaccharide structure mimic to chitin [2] and inhibits all family 18 chitinases, which hydrolyze chitin and are widely present in nature [3]. Allosamidin has been used to investigate the physiological role of chitinases involved not only in chitin-containing organisms, but also in non-chitin-containing ones. In the former case, it inhibits cell separation in yeast [4], transmission of the malaria parasite [5] and insect molting [1]. In the latter case, inhibition of acidic mammalian chitinase by allosamidin causes a decrease in asthmatic Th2 inflammation [6].

With respect to allosamidin and its producer, we found a phenomenon that chitinase production in a chitin medium by the producer was activated by addition of allosamidin exogenously [7]. This seems to indicate that allosamidin has a physiological role in its producing *Streptomyces* because chitinase production is very important for the bacterial growth in soil where chitin originated mainly from insect cuticle and fungal cell walls is a major nutrient source. In this report, we will provide strong evidence to prove this hypothesis by describing detailed allosamidin's effects on chitinase production and growth of its producer and identification of the chitinase whose production was promoted by allosamidin as well as a novel phenomenon of allosamidin release from the mycelia of its producing microbe by responding to chitin.

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Materials and Methods

Culture

Streptomyces sp. AJ9463, an allosamidin producer [8], was maintained on a Bennet agar medium consisting of glucose 1%, peptone 0.2%, meat extract 0.1%, and yeast extract 0.1%, pH 7.2, at 30°C and subcultured at about monthly intervals. Spores of the strain were inoculated into a Bennet medium (100 ml) in a 500-ml Erlenmeyer flask, and the flask was incubated at 30°C and 150 rpm on a rotary shaker for 46 hours. This preculture was used for all main cultures in this study. In the experiments with a chitin medium which consists of colloidal chitin 0.2%, KCl 0.05%, K₂HPO₄ 0.1%, MgSO₄·7H₂O 0.05% and FeSO₄·7H₂O 0.001%, (NH₄)₂SO₄ 0.2%, pH 7.2, the preculture (1.4 ml) was transferred into the chitin medium (70 ml) in a 500-ml Erlenmeyer flask, and the flask was incubated at 30°C and 120 rpm on a rotary shaker. We previously used slightly different culture conditions from those used in this study for the main culture of strain AJ9463 in the chitin medium, in which culture was done in a 500-ml Sakaguchi flask at 120 rpm instead of a 500-ml Erlenmeyer flask at 150 rpm. Analysis of the *N*-terminal amino acid sequence of 65 kDa protein was done with the culture filtrate obtained under this previously used culture condition. Allosamidin was dissolved in 0.1 M acetic acid, and each solution after being passed through a 0.25 μm sterile filter before use was added to the chitin medium or an inorganic salt solution at the beginning of culture.

Chitinase Assay

Chitinase assay was performed using 4-methylumbelliferyl-*N,N',N''*-triacetyl chitotrioside [4MU-(GlcNAc)₃] (Sigma-Aldrich) as a substrate according to the method of Kuranda *et al.* [9]. A solution of 25 μM 4MU-(GlcNAc)₃ in *N,N*-dimethylformamide was diluted five hundreds times with distilled water, and the resulting solution (40 μl) was added to the mixture of the culture filtrate (0.5, 2 or 4 μl) in each experiment and the 50 mM citric acid - 50 mM Na₂HPO₄ buffer (pH 5.0) (100 μl). After incubating the solution mixture at 28.5°C for 60 minutes, the reaction was stopped by addition of 1.0 M glycine - NaOH buffer (pH 10.5) (2.5 ml), and the liberated 4-methylumbelliferone was measured with a fluorescence spectrometer (excitation at 360 nm, emission at 450 nm). The value of fluorescence strength was used for calibration of relative chitinase activity.

Activity Staining

The culture supernatant (10 ml) in each experiment was

concentrated by ultrafiltration (10,000 cut membrane, AMICON) to 4 ml. Acetone (12 ml) was added to the concentrated solution to afford a precipitate. The precipitate was collected by centrifugation (7,000 *g*, 30 minutes) and dissolved in distilled water (30 μl), followed by mixing with the electrophoresis sample buffer (30 μl). After being left overnight at room temperature, the mixture was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. After SDS-PAGE, the gel was washed with 0.1 M AcONa - AcOH buffer (pH 5.0) for 5 minutes and layered over the gel containing glycolchitin (0.01%) and polyacrylamide gel (7.5%) with or without allosamidin to transfer the proteins in the lower gel into the upper gel. The gels were left in 0.15 M AcONa - AcOH buffer (pH 5.0) with or without allosamidin at 37°C for 180 minutes to allow an enzyme reaction by changing the buffer solution at 60 minutes intervals. The upper gel was stained by 0.01% fluorescent brightener 28 (Calcoflour, Sigma-Aldrich) in 0.1 M Tris-HCl (pH 8.8) buffer for 5 minutes at room temperature and washed overnight in water. The chitinase lytic bands on the gel were detected by a UV transilluminator.

Detection of Growth-promoting Activity of Allosamidin

Chitin contained in the culture was stained by 0.0005% fluorescent brightener 28 (Calcoflour, Sigma-Aldrich) and observed under a fluorescence microscope. The amount of ATP in each culture was measured by a luciferin-luciferase-based bioluminescence assay with a Lucifer LU plus kit (Kikkoman Co.) according to its protocol. Luminescence was measured by a luminometer (Luminescencer-JNR AB-2100, ATTO).

Chitinase Purification and Protein Sequencing

The preculture (1.4 ml) of strain AJ9463 prepared as described above was transferred into the chitin medium (70 ml) with allosamidin (2 μM) in a 500-ml Sakaguchi flask, and the flask was incubated at 30°C and 120 rpm on a rotary shaker for 24 hours. The culture filtrate (2.8 liters) was concentrated to 15 ml by ultrafiltration (10,000 cut membrane, AMICON) and added leupeptin (3 mg). This solution was put on a column which was packed with chitin powder (5 g, Wako) and preequilibrated with 50 mM Tris-HCl buffer (pH 9.0), and the column was left for 120 minutes at room temperature. After washing the column with 50 mM Tris-HCl buffer (pH 9.0) (30 ml), crude chitinases were eluted with 0.1 M acetic acid (90 ml). The 0.1 M acetic acid eluate was concentrated by ultrafiltration and desalted by cartridge filtration (MILLIPORE) to afford a crude chitinase solution (100 μl), which was subjected to 10% SDS-PAGE. After SDS-PAGE, proteins on the gel

were transferred to a polyvinylidene difluoride (PVDF) membrane (Clear Blot Membrane-p, ATTO) by electroelution at 100 mA for 63 minutes (per 85×90 mm membrane) with three transfer buffers (300 mM Tris containing 2% methanol and 0.05% SDS, 25 mM Tris containing 2% methanol and 0.05% SDS, and 25 mM Tris containing 40 mM 6-aminohexanoic acid, 2% methanol and 0.05% SDS). The PVDF membrane was stained with 0.2% Coomassie Brilliant Blue (CBB) R-250 in methanol for 5 minutes and bleached in 50% methanol containing 10% acetic acid for several minutes. Each band to be analyzed was cut from the membrane and subjected to the *N*-terminal amino acid sequencing with a protein sequencer (Model 492HT, Applied Biosystems), affording the sequences of 65 kDa protein (ATSATASVKKSDWGSFE), 52 kDa protein (GGSVPGDNAPSAPGTP), 47 kDa (AVTDTSAKLSWTAATD), 46 kDa protein (GGGGNPDPGTGGKVNLYFYTD) and 105 kDa protein (GGGGNPDP).

Cloning of *chi65* and the 5' Upstream Region of *chi65*

A DNA fragment encoding a part of the *N*-terminal amino acid sequence was obtained by PCR with the genomic DNA of strain AJ9463 and degenerate oligonucleotide primers (F1, 5'-TCCGCCACSGCSTCSTTYGT-3'; R1, 5'-TCGAAGG-TCAASCCCCARTC-3'), which were designed based on *N*-terminal amino acid sequence of 65 kDa chitinase above mentioned. The 5' and 3'-flanking regions of the DNA fragment obtained were amplified with the Genome Walker genomic libraries of strain AJ9463 (Genome Walker Kit; CLONTECH) by two consecutive rounds of PCR using the adaptor primers (AP1, 5'-GTAATACGACTCACTATAG-GGC-3', and AP2, 5'-ACTATAGGGCACGCGTGGT-3') and two primers (jf1, 5'-GCCACSGCGTTCGTTTYGTCAA-GAA-3', and jf2, 5'-GCGTCGTTYGTCAAGAAGTCCG-A-3', for the nucleotide sequence of the 3'-flanking region with a stop codon for *chi65*; gsp-r1, 5'-CGGAGGGGAAG-TCCCCTCGAT-3', and gsp-r2, 5'-CGGTGGTGCCGGT-GTTCTTCA-3' for the nucleotide sequence of the 5'-flanking region with *chi65R* and a part of *chi65S*; sq-r11, 5'-GGTGATCCCACCGTGAT-3', and sq-r12, 5'-CCCGA-CCGTGATCAGGAA-3', for the nucleotide sequence of the remaining part of *chi65S*; chi65SU1-r, 5'-CACGGGCTCG-CCGCGGTCTACAT-3', and chi65SU2-r, 5'-GCTCGGCC-ACTCCACGATCCGCT-3', for the nucleotide sequence of the 5'-upstream region of *chi65S*.

Nucleotide Sequencing

Amplified cDNA was ligated into a pCR 2.1 vector (Invitrogen), which was transformed into INV α F' cells (Invitrogen) according to the manufacturer's procedure. Plasmid DNAs extracted by a QIAprep Spin Plasmid

Purification kit (QIAGEN) were used for the sequencing with a cycle sequence kit (Thermo Sequence Cy 5.5 dye terminator cycle sequence kit, Amersham Pharmacia Biotech). Nucleotide sequence analyses were carried out on a DNA sequencer (Long-Read Tower DNA sequencer, Amersham Pharmacia Biotech, or ABI PRISM™ 310 Genetic Analyzer, PE Biosystems).

Allosamidin Release from Mycelia

The preculture (2.0 ml) of strain AJ9463 described above was inoculated into a Bennet medium (100 ml) in a 500-ml Erlenmeyer flask, and incubation was done at 30°C and 150 rpm on a rotary shaker for 120 hours. Mycelia was collected by centrifugation (3,000 *g*, 5 minutes) and washed with distilled water three times. The washed mycelia containing allosamidin (0.7 μ g) was resuspended in an inorganic salt solution (50 ml) consisting of KCl 0.05%, K₂HPO₄ 0.1%, MgSO₄·7H₂O 0.05%, FeSO₄·7H₂O 0.001%, (NH₄)₂SO₄ 0.2%, pH 7.2, or in a chitin medium (50 ml), and the suspension was incubated at 30°C and 150 rpm on a rotary shaker for 24 hours. The amounts of allosamidin in both the mycelia and supernatant were quantified by HPLC [8].

Results

Effect of Allosamidin on Chitinase Production

We used an allosamidin-producing strain *Streptomyces* sp. AJ9463 in this study since the genetic system of the strain has been established [10]. Figure 1 shows dose-response of allosamidin's effect on the chitinase production by strain AJ9463 when the strain was cultured in a chitin medium containing chitin as the sole carbon source. Allosamidin enhanced the chitinase activity of the culture filtrate dose-dependently up to 2 μ M (Fig. 1A). Clear enhancement was observed even at 60 nM. The patterns of proteins and chitinases in each culture filtrate were shown in Fig. 1B and 1C, which were detected by silver staining and activity staining after SDS-PAGE, respectively. Two bands at 105 kDa and 46 kDa with chitinase activity were detected in all culture filtrates, and the staining density of both bands increased in a dose-dependent manner. The presence of the two bands in the culture filtrate of a control without allosamidin indicates that they were main chitinases produced by strain AJ9463 in the chitin medium. The two chitinases were inhibited by allosamidin at high concentrations of more than 10 μ M (Fig. 2).

Growth-promoting Activity of Allosamidin

In the chitin medium, chitinase production is necessary for

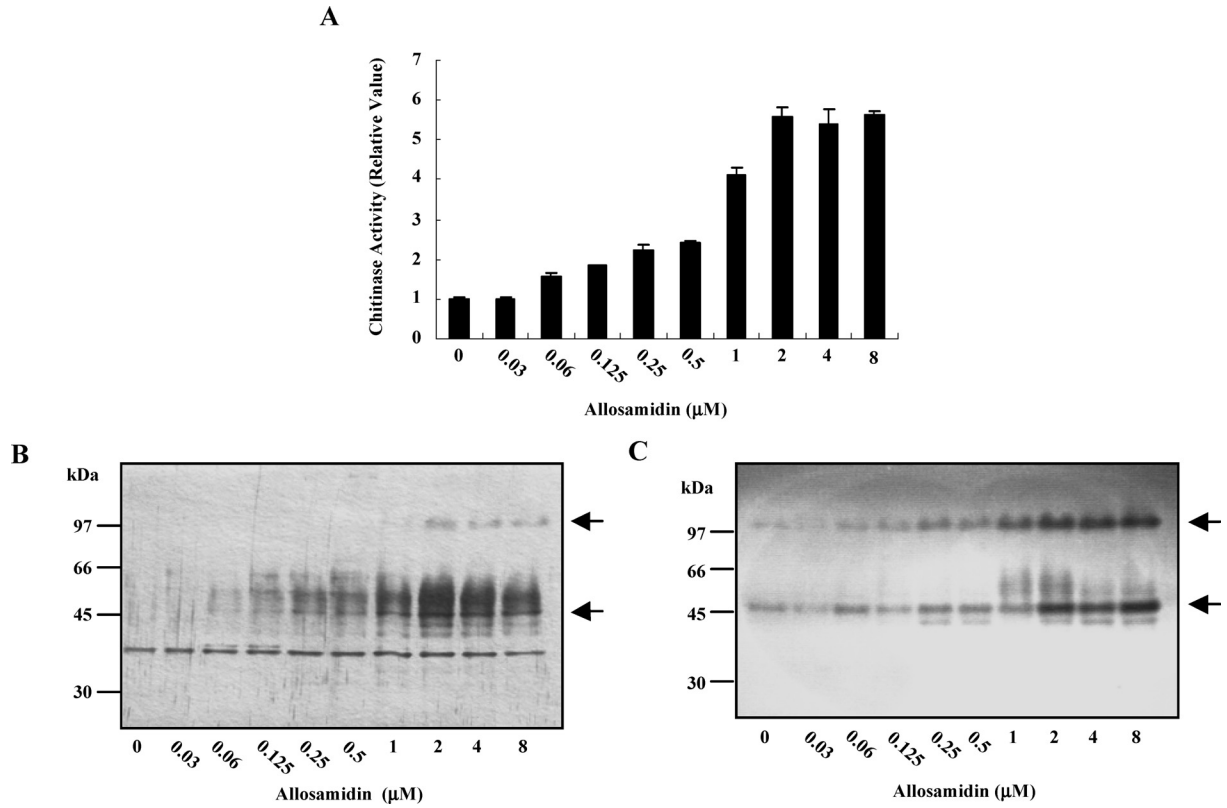


Fig. 1 Effects of allosamidin on chitinase production of *Streptomyces* sp. AJ9463.

Chitinase activity of the culture filtrate after 24 hours of cultivation in a chitin medium with allosamidin was measured with 4-methylumbelliferyl-*N,N,N'*-triacetyl chitotrioside [4MU-(GlcNAc)₃] as a substrate (A). Proteins and chitinases in each culture filtrate were analyzed by silver staining (B) and activity staining (C). In (A), the values of relative chitinase activity were calibrated based on the activity of a control without allosamidin and means±S.E. were shown (N=3). Arrows in (B) and (C) indicate the bands of 105 kDa and 46 kDa proteins.

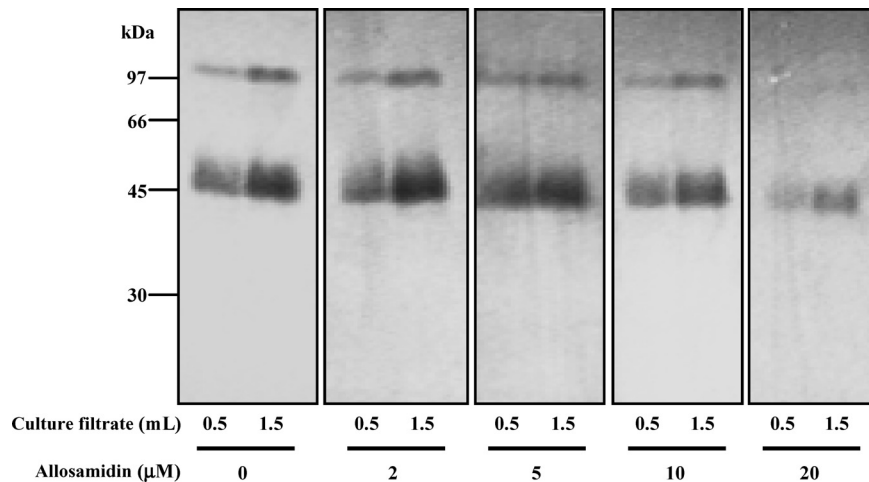


Fig. 2 Inhibition of 46 kDa and 105 kDa chitinase by allosamidin.

After cultivation of strain AJ9463 in a chitin medium for 24 hours, chitinase activity of the culture filtrate (0.5 or 1.5 ml) obtained was detected by activity staining using the chitin- and allosamidin (0~20 μM)-containing gel.

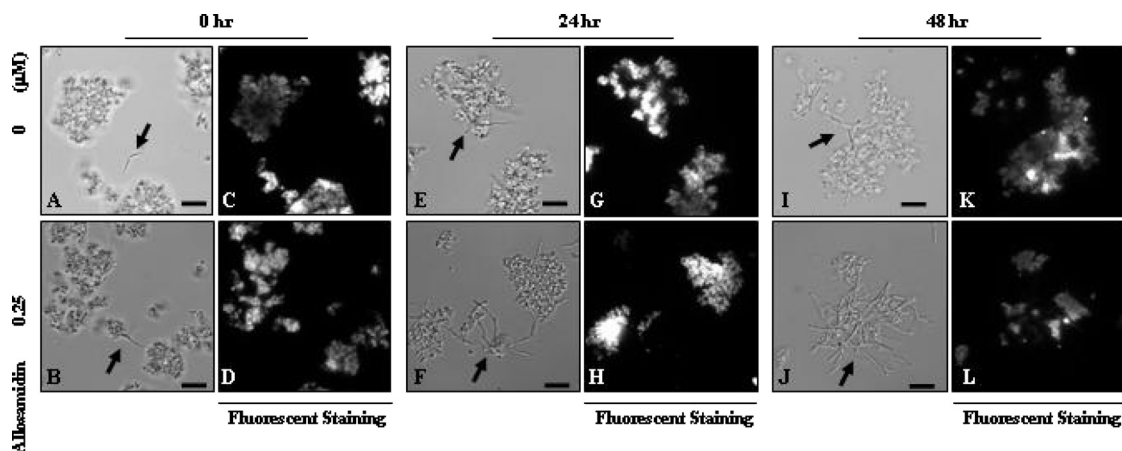


Fig. 3 Growth-promoting activity of allosamidin.

Strain AJ9463 was cultured in a chitin medium with (B, D, F, H, J and L) or without allosamidin (A, C, E, G, I and K) for 0 hour (A, B, C and D), 24 hours (E, F, G and H) or 48 hours (I, J, K and L). Each pair of A and C, B and D, E and G, F and H, I and K, or J and L is the same preparation. Mycelia, which are indicated by arrows, and chitin particles in each culture broth were observed under a microscope (A, B, E, F, I and J) and a fluorescence microscope by Calcofluor staining for detection of chitin (C, D, G, H, K and L), respectively. The pattern shown in each photograph was observed over the corresponding culture broth. Scale bar, 10 μm .

bacterial growth, and the enhancement of chitinase activity would lead to promoting the growth by accelerating chitin degradation. In fact, mycelia of strain AJ9463 grew much better in the presence of allosamidin compared to those of a control without allosamidin as shown in Fig. 3. At that time, the amount of chitin particles remaining in each culture broth, which was shown by Calcofluor staining (Fig. 3), indicated that chitin in the culture broth with allosamidin was degraded faster than that in the control culture. ATP levels in culture broths with or without allosamidin were also measured (Fig. 4), which supported the growth-promoting effect of allosamidin. The activation of chitinase production by allosamidin and subsequent growth promotion may synergistically increase the chitinase activity in the culture filtrate as shown in Fig. 1.

Identification of the Chitinase Whose Production Is Promoted by Allosamidin

When strain AJ9463 was cultured under previously used condition, the pattern of proteins produced in the culture filtrate was different from that under the condition used for experiments in Fig. 1 (1B and 5A). The strength of four bands at 105, 65, 52 and 47 kDa on SDS-PAGE of the culture filtrate obtained under the previous condition was mainly enhanced by addition of allosamidin (Fig. 5A). Among them, chitinase activity was detected at 105, 65 and 52 kDa bands by activity staining (Fig. 5B). We first analyzed the *N*-terminal amino acid sequence of the 65 kDa protein after purification of the crude culture filtrate with a chitin column as shown in Fig. 6 and obtained the gene

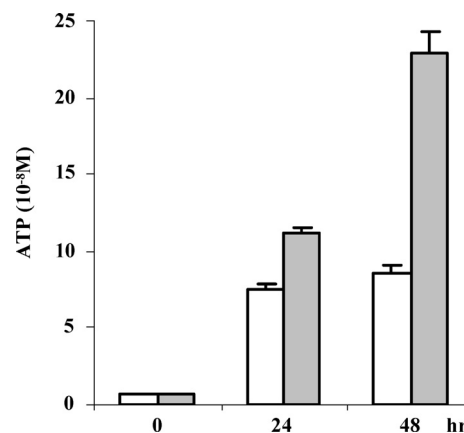


Fig. 4 Growth-promoting activity of allosamidin estimated from ATP amounts.

Strain AJ9463 was cultured in a chitin medium with (gray bars) or without (white bars) allosamidin (0.25 μM). ATP amount in each culture broth at 0, 24 or 48 hours cultivation time was measured with a luciferin-based bioluminescence assay kit.

encoding it, named *chi65* (AB239767), based on the sequence. The amino acid sequence deduced from the nucleotide sequence of *chi65* had high homology to known family 18 chitinases of *Streptomyces* such as chitinase 63 of *S. plicatus*, chitinase C of *S. coelicolor* and chitinase C of *S. lividans* [11]. Further analysis of the *N*-terminal amino acid sequences of 52 and 47 kDa proteins in Fig. 5A and 46 kDa protein in Fig. 1B showed that these three proteins also originated from the same gene, *chi65*. The *N*-terminal positions of these proteins are schematically

shown in Fig. 7. Analysis of the *N*-terminal amino acid sequence of 105 kDa protein revealed that the protein also originated from *chi65*, suggesting a dimer structure of 105 kDa protein.

A DNA fragment containing *chi65* was obtained and its nucleotide sequence revealed the presence of two genes, named *chi65S* and *chi65R* (AB239767), at the 5'-upstream region of *chi65*. The amino acid sequences deduced from the nucleotide sequences of the genes indicated that *chi65S* and *chi65R* encoded a sensor histidine kinase and a response regulator, respectively, constructing a typical two-component regulatory system.

Allosamidin Release from the Mycelia by Responding to Chitin

In all allosamidin-producing strains we isolated, most of the allosamidin produced has been obtained from mycelia cultured in a chitin-free medium such as a Bennet medium [12]. To know the mechanism of allosamidin transfer among cells, its localization in the culture broth was investigated with the chitin medium. Strain AJ9463 was

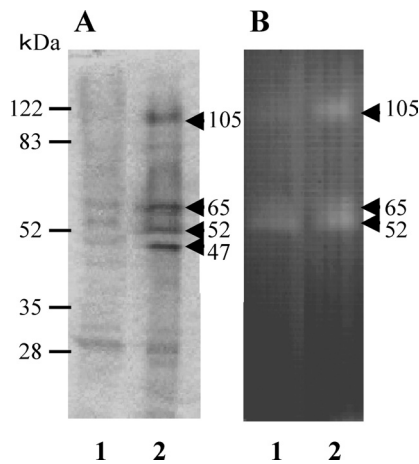


Fig. 5 The pattern of proteins produced by strain AJ9463 under the culture conditions previously used.

Proteins and chitinase activity of the culture filtrate after 24 hours cultivation in a chitin medium with (lane 2) or without (lane 1) allosamidin ($2 \mu\text{M}$) were detected by CBB staining after SDS-PAGE (A) and by activity staining on chitin-containing gel (B), respectively.



Fig. 7 Schematic representation of chitinases encoded by *chi65*.

Positions of *N*-terminal residue of 65, 52, 47 and 46 kDa proteins are indicated by solid arrows. SS, signal sequence; CB domain, chitin-binding domain; FT III domain, fibronectin type III domain.

cultured in a Bennet medium to obtain the mycelia which contained allosamidin. When the mycelia were collected and shaken in an inorganic salt solution, most of the allosamidin was maintained in the mycelia after incubation (Fig. 8). However, more than 80% of the allosamidin was recovered from the culture filtrate when the mycelia were incubated in the chitin medium. In the chitin medium, allosamidin production by strain AJ9463 starts at around 72 hours of cultivation and reaches a few hundred nM in the culture filtrate after 120 hours of cultivation.

Discussion

The variety of secondary metabolisms present in microorganisms suggests that they have been developing them during evolution and each secondary metabolite might have a significant role in its producer under

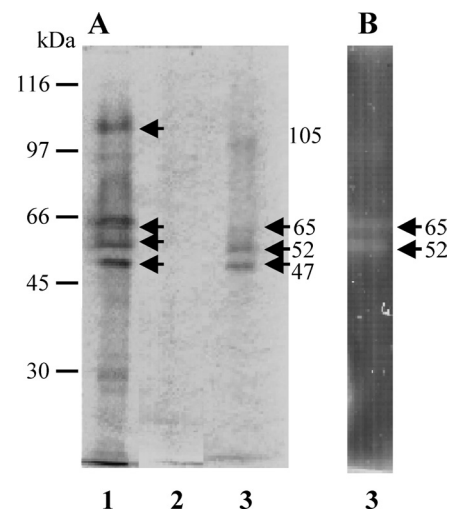


Fig. 6 Partial purification of chitinases by chitin column.

Culture filtrate after 24 cultivation in a chitin medium with allosamidin ($2 \mu\text{M}$) was absorbed on a chitin column and eluted with 50 mM Tris-HCl (pH 9.0) and 100 mM acetic acid (pH 3.0) successively. Each fraction (lane 1, culture filtrate; lane 2, 50 mM Tris-HCl eluate; lane 3, 100 mM acetic acid eluate) was electrophoresed and stained by CBB (A). Chitinase activity of the 100 mM acetic acid fraction was detected by activity staining (B).

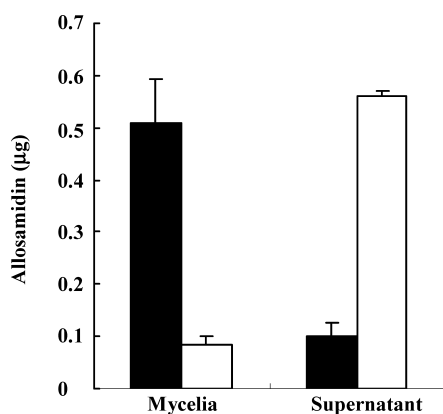


Fig. 8 Allosamidin release from mycelia.

Strain AJ9463 was cultured in a Bennet medium to obtain mycelia containing allosamidin (0.7 µg), which were incubated in an inorganic salt solution (black bars) or in a chitin medium (white bars). After 24 hours of incubation, the amounts of allosamidin in both the mycelia and supernatant were quantified by HPLC. Means ± S.E. were shown (N=3).

some circumstances. The role of antibiotic production is presumable but has not been proved. It is entirely unknown why microorganisms produce many other compounds without antibiotic activity such as enzyme inhibitors. In this study, to present a model of a physiological role of a microbial secondary metabolite, we focused on action of the chitinase inhibitor allosamidin on chitinase production in its producing *Streptomyces*.

Allosamidin can strongly enhance the chitinase production of strain AJ9463 dose-dependently up to a few µM. The effective concentration of allosamidin on chitinase production may be physiologically significant because the concentration of allosamidin produced in the culture broth was usually around the range of a few hundred nM to a few µM [12]. Moreover the fact that the enzymatic activity of the chitinase whose production is enhanced by allosamidin was not inhibited under the presence of a few µM of allosamidin suggests that the allosamidin's effect on chitinase production is not disturbed by its chitinase inhibitory activity at low concentrations effective on chitinase production.

In many cases, *Streptomyces* chitinase has been found in the culture broth as a degradation product of a protein encoded by a chitinase gene [11, 13]. Furthermore, some cases are known that multiple degradation proteins with chitinase activity are formed from a chitinase protein [14]. Therefore, multiplicity of chitinase proteins detected in the culture filtrate of strain AJ9463 is not an extraordinary case although it is not clear why the degradation pattern was different between two culture conditions. The fact that all

chitinases mainly produced in the chitin medium originated from a gene *chi65* may indicate importance of the chitinase gene for growth of the strain in chitin-rich environment.

In the two-component regulatory system of CHI65S and CHI65R, a signal molecule existing outside of the cells may attach the sensor moiety of CHI65S, a membrane protein with five membrane-spanning domains, and the signal may activate CHI65R, a DNA binding protein with a helix-turn-helix domain, by a phosphorylation relay system observed in usual two-component regulatory systems [15]. The activated CHI65R would regulate *chi65* expression. Many chitinase genes of *Streptomyces* have been obtained, but in only two cases, *chi40* of *S. thermoviolaceus* [16] and *chiC* of *S. coelicolor* [17], homologs of *chi65S* and *chi65R* are present in the upstream region of each chitinase gene. Although *S. lividans* transformed with a DNA fragment containing *chi40* and two genes encoding the two-component system could produce CHI40 in the presence of chitin and a gene disruption experiment suggested that the two-component system may regulate the *chiC* expression in *S. coelicolor*, detailed mechanism for the regulation including a ligand of the sensor moiety has not been clarified in both cases.

We found a novel phenomenon that allosamidin is released from the mycelia when chitin is present in a medium. This indicates that chitin or some other factors such as degradation products of chitin can release allosamidin from the mycelia, which is very important for allosamidin's action as a signal molecule and may validate our experiments in which allosamidin was added exogenously into the chitin medium.

From this study, we can imagine the following physiological role of allosamidin in its producing *Streptomyces*. Since the growing phase of mycelia in *Streptomyces* is not synchronized, allosamidin production would start in some part of mycelia at the middle growing phase [12]. The allosamidin produced would be released to the outside of the mycelia in the presence of chitin and transported to any part of mycelia in the allosamidin-producing strain itself or other allosamidin-producing strains. It would activate the chitinase production through the two-component regulatory system, which would further promote growth of the allosamidin-producing strains in a chitin-rich environment. This is a novel model demonstrating that a secondary metabolite of *Streptomyces* with a specific biological activity shows clear physiological activity toward its producer. Studies to investigate the mechanism for regulation of chitinase production by allosamidin through a two-component regulatory system are described in the following paper.

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