Allosamidin, a chitinase inhibitor produced by *Streptomyces*, acts as an inducer of chitinase production in its producing strain

By Eiyu NAKANISHI, Susumu OKAMOTO, Hiroshi MATSUURA, Hiromichi NAGASAWA, and Shohei SAKUDA[†]

Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657
(Communicated by Saburo TAMURA, M. J. A., May 14, 2001)

Abstract: Allosamidin is a chitinase inhibitor produced by *Streptomyces*. Allosamidin-producing *Streptomyces* is frequently found in soil. Allosamidin is a hydrophilic compound, but is found only in mycelia of its producer, and exogenous allosamidin is adsorbed on mycelia of its producing strain. We found that exogenous addition of allosamidin to a culture medium with colloidal chitin as a sole carbon source can strongly accelerate the chitinase production by an allosamidin-producing *Streptomyces* at low concentrations such as 1 μM. On the other hand, allosamidin depressed the chitinase activity in the culture of *Streptomyces lividans*, an allosamidin-nonproducing microbe. These results suggest that allosamidin has a physiological role in its producer as an inducer of chitinase production, which may be useful for chitin utilization by the producer in chitin-rich environment such as in soil.

Key words: Allosamidin; chitinase; *Streptomyces*; secondary metabolite.

Introduction. In 1986, we isolated allosamidin from mycelial extracts of *Streptomyces* as a first chitinase inhibitor.[1),2)] Allosamidin has a unique pseudotrisaccharide structure consisting of two units of *N*-acetyl-D-allosamine and one unit of an aminocyclitol derivative having a cyclopentanoid skeleton fused with an aminooxazoline ring (Fig. 1).[2)] Chitinases are classified into the two families, 18 and 19.[3)] Family 18 chitinases are present in nature much more widely than family 19 chitinases, and they are thought to play an important role for growth of each organism containing them. Allosamidin strongly inhibits all family 18 chitinases, but does not inhibit family 19 chitinases, and shows interesting biological activities against a variety of chitin-containing organisms.[4)] The structure of allosamidin is a mimic of chitin and its aminocyclitol derivative moiety may bind an active center of family 18 chitinases, which was shown by X-ray analysis of an allosamidin-chitinase complex.[5)]

During the course of our study on allosamidin, we met some interesting phenomena regarding allosamidin production and its producer as follows: (1) in our screening search for new chitinase inhibitors, allosamidin-producing *Streptomyces* was frequently found in soil,[6)] (2) allosamidin is a hydrophilic compound, but it is produced only in mycelia of its producing microbe, and allosamidin was adsorbed on mycelia of allosamidin-producing strain when it was added to a medium exogenously, (3) chitinase activity was detected in the culture filtrate of all allosamidin-producing *Streptomyces* we isolated,[7)] and (4) concurrent decrease in both of chitinase and allosamidin production was observed in a strain obtained from a stocked slant. These observations prompted us to investigate a physiological role of allosamidin in its producer.

In general, physiological roles of secondary metabolite production in its producing organisms are not clear. *Streptomyces* is one of the most important microorganisms as a producer of a variety of useful secondary metabolites. But, it is still unknown why *Streptomyces* produces secondary metabolites such as antibiotics or enzyme inhibitors. Allosamidin and its producing *Streptomyces* might be useful tools to answer this question.

To obtain clues to the physiological role of allosamidin, we previously focused chitinases produced by an allosamidin-producing strain, *Streptomyces* sp. AJ9463, but it became clear that they do not have any specific characters such as allosamidin-insensitivity in the family 18 chitinase produced by the strain.[7)] This

[†)] Correspondence to: S. Sakuda.

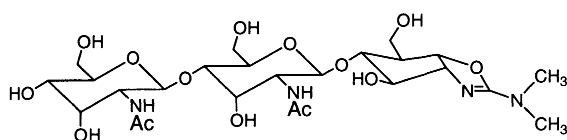

Fig. 1. Structure of allosamidin.

time, we examined the effect of allosamidin on chitinase production by strain AJ9463, and found that allosamidin could strongly accelerate the production. This paper describes the preliminary analysis of the physiological role of allosamidin as an inducer of chitinase production in its producer, which may be a precious case that a role of microbial secondary metabolite in its producing strain becomes clear.

Materials and methods. *Culture*. One strain of *Streptomyces* sp. AJ9463 was used in this study. Spores of the strain (100 µL) were inoculated into Bennet medium (100 mL), which consisted of glucose (1%), peptone (0.2%), meat extract (0.1%), and yeast extract (0.1%) (pH 7.2), for preculture in a 500-mL Erlenmeyer flask, and the flask was incubated at 30 °C and 150 rpm on a rotary shaker for 46 h. This culture (1.4 mL) was transferred into a medium (70 mL) consisting of colloidal chitin (0.15%), KCl (0.05%), K_2HPO_4 (0.1%), $MgSO_4$ (0.05%), and $FeSO_4$ (0.001%) (pH 7.0) in a 500-mL Sakaguchi flask for the main culture, and the flask was incubated at 30°C and 120 rpm on a reciprocating shaker for 48 h. Allosamidin, which was obtained from mycelial methanol extracts of strain AJ9463 according to the procedure described previously,[8] and *N*-acetylchitobiose were dissolved in a small amount of 0.1 M acetic acid and water, respectively, and each solution was passed through a sterile Millipore filter before addition. The solution (< 20 µL) was added in one portion to each 500 mL flask containing the medium (70 mL) just before the beginning of the main culture. *Streptomyces lividans* TK21 was cultured under the same conditions as used in cultivation of strain AJ9463 described above.

Chitinase assay. Chitinase assay was performed with 4-methylumbelliferyl-*N,N′,N″*-triacetylchitotrioside as a substrate according to the method of Kuranda *et al.*[9] After lyophilization of each culture filtrate (100 µL) of 11, 16, 24, 32, 41 and 48 h of cultivation, the residue was dissolved in 600 µL of 50 mM citric acid - Na_2HPO_4 buffer (pH 5.0). This crude enzyme solution (110 µL) was mixed with 10 µL of 0.1 M acetic acid with or without allosamidin (10 µg) and 40 µL of 25 mM 4-methylumbelliferyl-*N,N′,N″*-triacetylchitotrioside in 50 mM citric acid - Na_2HPO_4 (pH 5.0) buffer. The reaction solution was incubated at 28 °C for 60 min, and 2.5 mL of 1.0 M glycine - NaOH buffer (pH 10.5) was added to the solution. The liberated 4-methylumbelliferone was measured with a fluorescence spectrophotometer (excitation at 350 nm, emission at 440 nm). The value of fluorescence strength was used for calibration of relative chitinase activity.

Activity staining. Activity staining to detect the chitinase activity on a gel was performed according to the procedure of Koga *et al.*[10] After 48 h cultivation of strain AJ9463 with or without allosamidin (10 µM), the obtained culture filtrate (25 mL) was mixed with 10 µL of leupeptin solution (1.25 mg/mL in water) and 1.9 mg of 4-(2-aminoethyl)-benzenesulfonyl fluoride. After lyophilization of the solution, the residue was dissolved in 3 mL of distilled water and dialyzed against distilled water. The dialyzate was lyophilized again and dissolved in 250 µL of distilled water. This crude enzyme solution (6 µL) was put on the polyacrylamide gel for electrophoresis. After electrophoresis under native conditions, the gel was layered over the chitin-containing gel, which contained agarose (2.0%) and glycolchitin (0.05%) in 100 mM AcONa-AcOH buffer (pH 5.0), to transfer the proteins in the upper gel into the lower gel. The gels were left at 37 °C for 120 min to allow the enzyme reaction. The chitin-containing gel was stained by 0.1% Congo Red aqueous solution for 60 min at room temperature, and immersed in 1.0 M NaCl for 60 min. The chitinase lytic bands on the gel were observed as white area.

Results and discussion. *Streptomyces* sp. AJ9463, a high producer of allosamidin, was used throughout this study. Provided that allosamidin has an effect on chitinase production by strain AJ9463, it is expected that allosamidin exogenously added into a culture might affect chitinase production by the strain. Since we confirmed that exogenous allosamidin added into the culture of strain AJ9463 was adsorbed on mycelia of the strain by the experiment with ^{14}C-labeled allosamidin,[11] exogenous allosamidin might not inhibit chitinases outside of the mycelia newly produced after its addition. To examine the effect of allosamidin on chitinase production, we planned experiments with a medium containing colloidal chitin as a sole carbon source. In such a medium, chitinase production may be necessary for growth of the microorganism.

Strain AJ9463 was cultured in a 500 mL-Sakaguchi flask containing the colloidal chitin medium (70 mL) with or without allosamidin (2 µM) for 2 days. Chitinase

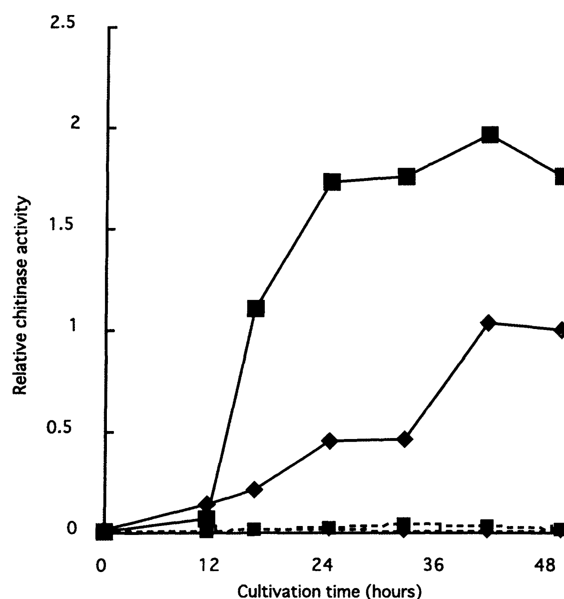

Fig. 2. Effects of allosamidin on the chitinase production by *Streptomyces* sp. AJ9463. Symbols: —◆—, cultured without allosamidin (control); —■—, cultured with allosamidin (2 μM); - -◆- -, cultured without allosamidin and chitinase activity was measured in the presence of allosamidin; - -■- -, cultured with allosamidin (2 μM) and chitinase activity was measured in the presence of allosamidin. The values of relative chitinase activity were calibrated based on the activity of control at 48 h of cultivation. Each value represents mean of duplicated experiments.

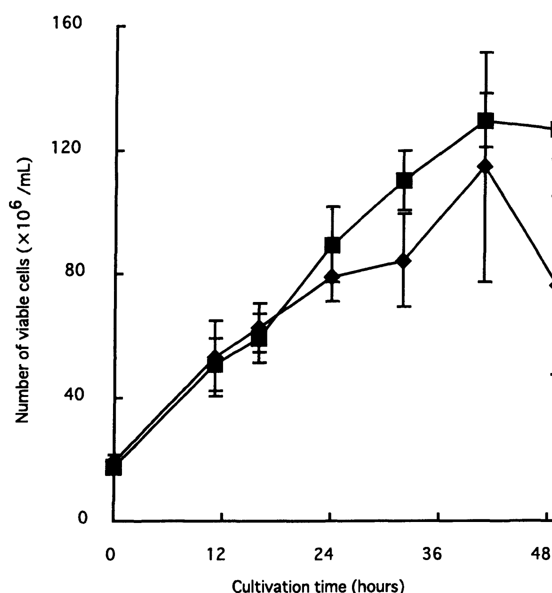

Fig. 3. Course of numbers of viable cells of *Streptomyces* sp. AJ9463. Symbols: —◆—, cultured without allosamidin; —■—, cultured with allosamidin (2 μM). Each value represents mean of triplicated experiments and vertical bar represents standard deviation.

activity in the culture filtrate was measured using 4-methylumbelliferyl-*N*,*N*′,*N*″-triacetylchitotrioside as a substrate. Fig. 2 shows the changes in the activity during the cultivation. In control culture, chitinase production started around 11 h of cultivation and the chitinase activity increased gradually. When allosamidin was added to the culture, chitinase production started around 11 h of cultivation as in control, but the activity increased rapidly and was maintained at higher levels than that in the control during the cultivation. When allosamidin was added to the culture filtrate at the enzyme assay to inhibit family 18 chitinases involved in the culture filtrate, chitinase activity drastically decreased in both culture filtrates from control and culture with allosamidin (Fig. 2). This indicated that most of chitinase activity in the culture filtrate is attributed to the action of family 18 chitinases. Fig. 3 shows changes in numbers of viable cells of the microbe during the cultivation. Clear effect of allosamidin on the numbers was not observed at all cultivation times.

Table I shows the effects of allosamidin on chitinase production by strain AJ9463 at various concentrations. Allosamidin could clearly accelerate the chitinase production even at a concentration of 1 μM (0.6 mg/L). *N*-Acetylchitobiose is known as an inducer of chitinase production in *Streptomyces lividans*.[12] Chitinase production by strain AJ9463 was also accelerated by *N*-acetylchitobiose, but its effect is weaker than allosamidin as shown in Table I.

Table I. Effects of allosamidin and *N*-acetylchitobiose on chitinase production by *Streptomyces* sp. AJ9463

Compound	Concentration (μM)	Relative chitinase activity[a]
A[b]	1	1.8
A	2	1.9
A	4	2.0
A	8	2.1
C[c]	8	1.6
C	16	1.7

[a] Chitinase activity in the culture filtrate after 48 h of cultivation was measured and normalized based on the value of control cultured without allosamidin or chitobiose as 1.0. Each value represents mean of duplicated experiments. [b] Allosamidin. [c] *N*-Acetylchitobiose.

Next, the chitinase activity in the culture filtrate of strain AJ9463 cultured with or without allosamidin was analyzed by using the activity staining. Crude enzyme was prepared from the culture filtrate obtained after 48 h cultivation, and chitinase activity of the crude enzyme was detected by activity staining after gel electrophoresis. As shown in Fig. 4, much stronger chitinase activity

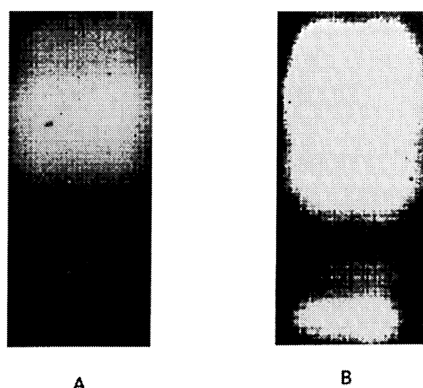

Fig. 4. Effects of allosamidin on chitinase production by *Streptomyces* sp. AJ9463 detected on a chitin-containing gel by activity staining. Crude enzymes from the culture filtrates after 48 h cultivation without (A) and with (B) allosamidin (10 μM).

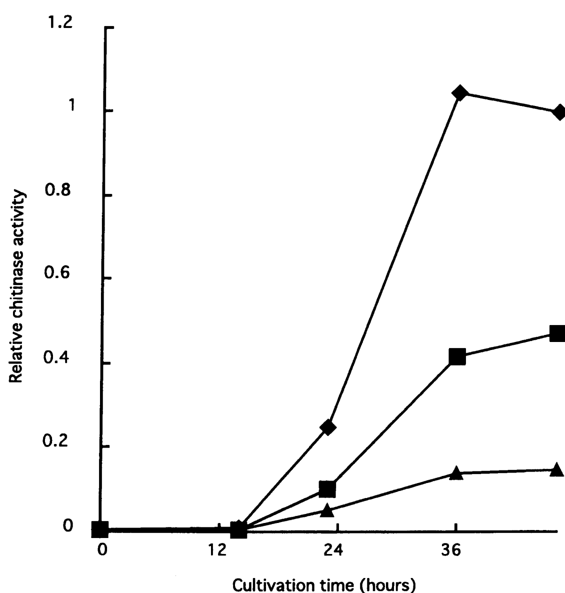

Fig. 5. Effects of allosamidin on chitinase activity in the culture of *Streptomyces lividans*. Symbols: —◆—, cultured without allosamidin (control); —■—, cultured with allosamidin (2 μM); —▲—, cultured with allosamidin (8 μM). The values of relative chitinase activity were calibrated based on the activity of control at 48 h of cultivation. Each value represents mean of duplicated experiments.

was observed on the gel of the sample from culture with allosamidin than control. Since multiple bands were observed on the gel, production of multiple chitinases may be induced by allosamidin.

Finally, the effect of allosamidin on chitinase production by *S. lividans* was examined. *S. lividans* produces multiple chitinases,[12] and allosamidin production is not detected in its mycelia. *S. lividans* was cultured in the colloidal chitin medium with or without allosamidin under the same conditions as used in cultivation of strain AJ9463. Fig. 5 shows the changes in chitinase activity in the culture filtrate during 48 h cultivation. Allosamidin clearly caused decrease of the activity dose-dependently. This suggests that allosamidin can not activate chitinase activity in cultures of all *Streptomyces*.

In this study, we found that allosamidin accelerated chitinase production in an allosamidin-producing strain. This fact may suggest that allosamidin has a physiological role in its producer. Allosamidin is a typical secondary metabolite produced by a microorganism. We believe that this allosamidin case is a good model to study why microorganisms produce secondary metabolites. Work to investigate the molecular mechanism for chitinase induction by allosamidin is now in progress.

References

1) Sakuda, S., Isogai, A., Matsumoto, S., and Suzuki, A. (1987) J. Antibiot. **40**, 296-300.
2) Sakuda, S., Isogai, A., Matsumoto, S., Suzuki, A., and Koseki, K. (1986) Tetrahedron Lett. **27**, 2475-2478.
3) Henrissat, B. (1991) Biochem. J. **280**, 309-316.
4) Sakuda, S. (1996) In Chitin Enzymology (ed. Muzzarelli, R. A. A.). vol. 2, Arec Edizioni, Italy, pp. 203-212.
5) Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B., and Dijkstra, B. W. (1995) Biochemistry **34**, 15619-15623.
6) Nishimoto, Y., Sakuda, S., Takayama, S., and Yamada, Y. (1991) J. Antibiot. **44**, 716-722.
7) Wang, Q., Zhou, Z.-Y., Sakuda, S., and Yamada, Y. (1993) Biosci. Biotech. Biochem. **57**, 467-470.
8) Zhou, Z.-Y., Sakuda, S., and Yamada, Y. (1992) J. Chem. Soc. Perkin Trans. 1, 1649-1652.
9) Kuranda, M. J., and Robbins, P. W. (1991) J. Biol. Chem. **266**, 19758-19767.
10) Koga, D., Funakoshi, T., Fujimoto, H., Kuwano, E., Eto, M., and Ide, A. (1991) Insect Biochem. **21**, 277-284.
11) Zhou, Z.-Y., Sakuda, S., Kinoshita, M., and Yamada, Y. (1993) J. Antibiot. **46**, 1582-1588.
12) Miyashita, K., Fujii, T., and Saito, A. (2000) Biosci. Biotech. Biochem. **64**, 39-43.