The Role and Characterization of β-1,3-Glucanase in Biocontrol of *Fusarium solani* by *Pseudomonas stutzeri* YPL-1

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An antifungal *Pseudomonas stutzeri* YPL-1 produced extracellular chitinase and β-1,3-glucanase that were key enzymes in the decomposition of fungal hyphal walls. These lytic extracellular enzymes markedly inhibited mycelial growth of the phytopathogenic fungus *Fusarium solani*. A chitinase from *P. stutzeri* YPL-1 inhibited fungal mycelial growth by 87%, whereas a β-1,3-glucanase from the bacterium inhibited growth by 53%. Furthermore, co-operative action of the enzymes synergistically inhibited 95% of the fungal growth. The lytic enzymes caused abnormal swelling and retreating on the fungal hyphal walls in a dual culture. Scanning electron microscopy clearly showed hyphal degradation of *F. solani* in the regions interacting with *P. stutzeri* YPL-1. In an *in vivo* pot test, *P. stutzeri* YPL-1 proved to have biocontrol ability as a powerful agent in controlling plant disease. Planting of kidney bean (*Phaseolus vulgaris L.*) seedlings with the bacterial suspension in *F. solani*-infested soil significantly suppressed the development of fusarial root-rot. The characteristics of a crude preparation of β-1,3-glucanase produced from *P. stutzeri* YPL-1 were investigated. The bacterium produced high levels of β-1,3-glucanase after 72 hr of incubation and a peak of its activity was detected after 2 hr of incubation. The enzyme had optimum temperature and pH of 40°C and pH 5.5, respectively. The enzyme was stable in the pH range of 4.5 to 7.0 and at temperatures below 40°C, with a half-life of 40 min at 60°C.

Key words: β-1,3-glucanase, chitinase, *P. stutzeri* YPL-1, *F. solani*, lysis, biological control

Hyperparasitism and lysis are the most important forms of biological control of soilborne plant pathogens by microbial antagonists. The mechanism appears to involve the enzymatic hydrolysis of chitin and glucan components of fungal hyphal walls. The lytic activity of bacterial and fungal antagonists is mainly due to the extracellular lytic enzymes chitinase and β-1,3-glucanase (7, 18).

β-1,3-glucanase is a semiconstitutive enzyme (5) that hydrolyzes β-1,3-glucans, important structural components of cell walls in many agronomically important pests including phytopathogenic fungi or in various yeasts. β-1,3-glucanase is widely distributed in bacteria (2, 4, 12, 15), fungi (8, 10, 11, 23), yeasts (6, 19, 22), and higher plants (1, 9).

Recently, the hydrolytic enzymes have received considerable attention because they may play a role in plant growth-promoting systems against plant pathogens. The enzymatic digestion or deformation cell wall components of these organisms by the enzymes could present an effective method for their biological control.

A *Pseudomonas stutzeri* YPL-1 was isolated and characterized on the basis of its ability to lyse the cell walls of the phytopathogenic fungi *Fusarium solani* causing root-rots leading to considerable losses in many important crops (13). *P. stutzeri* YPL-1 was found to liberate extracellular chitinase and β-1,3-glucanase, enzymes essential for the decomposition of fungal hyphal walls as a factor of biocontrol (13). In former studies, we previously described the characteristics of extracellular chitinase from *P. stutzeri* YPL-1 in order to investigate the detailed mode of lytic action (14).

In this paper, we describe (i) the role of β-1,3-glucanase in the biological control of *F. solani* by *P. stutzeri* YPL-1, (ii) the biocontrol effect against root-rot of kidney bean (*Phaseolus vulgaris* L.) caused by *F. solani in vivo* bioassay, and (iii) the characteristics of a crude preparation of the β-1,3-glucanase.

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Materials and Methods

Chemicals

Laminarin from *Eisenia arborea* was purchased from Tokyo Chemical Co. Chitin powder extracted from crab shell was purchased from Sigma Chemical Co. Colloidal chitin used in the chitinase assay was prepared by the method of Bemiller (3). All other chemicals were special grade products.

Microorganisms and culture conditions

Pseudomonas stutzeri YPL-1, originally isolated from rhizosphere in ginseng root-rot-suppressive soil, was used in this study (13). The bacterium was grown and maintained on nutrient agar (NA). *Fusarium solani*, which causes plant root-rot, was provided by the Korean Ginseng and Tobacco Research Institute and was grown on a potato dextrose agar (PDA). The microorganisms were lyophilized for long-term storage.

Enzyme preparation

The cell wall-degrading enzymes β-1,3-glucanase and chitinase were prepared from the culture supernatant of *P. stutzeri* YPL-1. For the preparation of β-1,3-glucanase, the bacterium was grown at 30℃ for 72 hr with aeration in a laminarin-peptone medium containing 0.5% glucose, 0.5% peptone, 0.2% laminarin, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% NaCl. The pH of the medium was adjusted to 6.8 prior to autoclaving. For the preparation of chitinase, the strain was grown aerobically in peptone medium containing 0.2% chitin at 30℃ for 84 hr. The cultures were centrifuged aseptically at 12,000 $\times g$ for 20 min at 4℃. The lytic enzymes were purified by ammonium sulfate precipitate fractionation.

Assay for enzyme activity

β-1,3-glucanase activity was assayed by monitoring the release of reducing sugar using laminarin as substrate according to the method of Somogyi-Nelson (20). The reaction mixture contained 0.3 ml of 1 M sodium acetate buffer (pH 5.5), 0.5 ml of 0.2% soluble laminarin, and 0.25 ml aliquots of appropriately diluted enzyme. One unit of β-1,3-glucanase activity was defined as the amount of the enzyme releasing 1 μmol of glucose per hour under these conditions. Chitinase activity was determined by measuring the release of N-acethyl-D-glucosamine (GlcNAc) from colloidal chitin as substrate by the method of Reissig *et al.* (21). The assay mixture consisted of 0.3 ml of 1 M sodium acetate buffer (pH 5.3), 0.5 ml of 0.2% colloidal chitin, and 0.25 ml of suitably diluted enzyme. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour during these conditions.

Protein assay

Protein concentration was determined spectrophotometrically at 280 nm or by the method of Lowry *et al.* (16) with bovine serum albumin as a standard.

Antifungal tests

The antifungal activities of extracellular chitinase or β-1,3-glucanase produced from *P. stutzeri* YPL-1 on the mycelial growth of *F. solani* were assayed according to the method of Lim *et al.* (13). Each of the lytic enzyme solutions from *P. stutzeri* YPL-1 was aseptically added to 2.64% potato dextrose broth (PDB) preincubated with *F. solani* for 3 days. The cultures were grown at 28℃ for 3 days. The fungal mycelia were collected on oven-dried preweighed paper (Whatman No. 2 filter paper) and dried at 105℃, and the dry weights were determined. The inhibition ratios were expressed relative to a control with water. To determine the antifungal activities of *P. stutzeri* YPL-1 and its mutants against *F. solani*, samples (5 μl, containing approximately 10^6 cells) from the overnight cultures of bacterial strains in nutrient broth (NB) were inoculated around 1 cm from the edge of plates and allowed to soak into the agar. An agar disk (5 mm in diameter) of *F. solani* inoculum from the leading edge of a culture of *F. solani* grown at 28℃ for 3 days on PDA was placed in the center of the plate. Plates were incubated at 28℃ and scored after 5 days by measuring the distance between the edges of the bacterial colonies and fungal mycelium.

NTG mutagenesis

P. stutzeri YPL-1 was mutated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment. The NTG mutagenesis procedure was based on the method of Miller (17).

Pot bioassay

To determine the efficacy of *P. stutzeri* YPL-1 as a biocontrol agent against root-rot disease caused by *F. solani*, kidney bean (*Phaseolus vulgaris* L.) seedlings were used as host plants in our study. Kidney bean seeds were germinated in a incubator at 30℃ for 2 days at 100% relative humidity. The bacterial suspension was prepared by growing in acrated NB at 30℃. The bacterial culture was centrifuged at 10,000$\times g$ for 10 min and then the cells were resuspended in sterile saline. The desired concentration (approximately 1×10^8 cells per ml) was obtained by adjusting the suspension according to the standard curve with a spectrophotometer (Hitachi U2000). The spore suspension of *F. solani* was prepared by growing in PDB at 28℃ for 7 days. The cultures

were sieved aseptically through 10 layers of sterile gauze, centrifuged, and washed five times in sterile saline. The spore suspension was air-dried and stored at 4℃ until used in pot bioassay.

Polypropylene boxes (50×50×50 mm) were filled up to two-thirds with field soil, and 25 seeds were placed in each box. A seed cover layer (one-third of the pot's depth) was infested with a preparation of *F. solani* (16~20 mg per g soil) and mixed with 5 ml of the bacterial suspension prepared as described above. Soil infested with the pathogen but not treated with bacteria served as a control. Plants were watered daily with 500 ml.

Scanning electron microscopy

Microscopic observations were carried out on the interacting regions of *F. solani* grown with *P. stutzeri* YPL-1 in a dual culture. The samples were fixed with 3% glutaraldehyde in a 0.2 M phosphate buffer (pH 6.5) for 3 hr, and washed with the same buffer for 15 min, fixed with 2% OsO_4 for 2 hr, and finally washed again with the buffer. The material was dehydrated with ethanol at 4℃ by using a series of steps for 10 min each. The specimens were dried in a Hitachi HCP-2 critical point drier with CO_2 as the carrier gas. The dried specimens were mounted on stubs with Television Tube Koat to prevent charging. The specimens were coated with gold palladium in an Ion Coater Giko IB-5 and observed with a scanning electron microscope (ISISS 103).

Results and Discussion

Antagonism by lytic action and biocontrol ability

Pseudomonas stutzeri YPL-1 released extracellular chitinase and β-1,3-glucanase which are key enzymes in the decomposition of fungal hyphal walls, when grown in a medium containing colloidal chitin, laminarin, or dried mycelium of *Fusarium solani* as carbon sources (Table 1). In several antifungal tests with the culture filtrates of *P. stutzeri* YPL-1, the antagonistic substances involved in fungal inhibition appeared to be heat-labile, macromolecular proteins (13). The results indicate that the antifungal mechanism of *P. stutzeri* YPL-1 against *F. solani* may involve a lysing agent such as a hydrolytic enzyme activity rather than a toxic substance or on antibiotic. This was confirmed by mutational analysis of a mutant *P. stutzeri* YPL-M122 that lacked the antifungal activities with the activities of both chitinase and β-1,3-glucanase (Table 2).

The cell walls of *F. solani* are composed mostly of 47% chitin, with 14% glucan (24). Therefore, it is likely that lysis of the cell walls is caused by the concerted action of β-1,3-glucanase with chitinase. A mutant *P. stutzeri* YPL-M153 that lacked both 67% of β-1,3-glucanase productivity and 100% of chitinase inhibited about 20% of the mycelial growth of *F. solani* inhibited by *P. stutzeri* YPL-1 (Table 2). A crude chitinase (10 μg/ml) produced from *P. stutzeri* YPL-1 inhibited fungal growth by 87% compared with that of the untreated control after 24 hr of incubation. A crude β-1,3-glucanase (17 μg/ml) from the bacterium inhibited fungal growth by 53% (Fig. 1). Although the antifungal activity of the chitinase was much higher than that of the β-1,3-glucanase, co-operative action of the crude enzymes inhibited fungal growth by 95%. The results indicate that the antifungal activity of *P. stutzeri* YPL-1 against *F. solani* was increased by the synergistic effect of concerted action by the enzymes. Accordingly, β-1,3-glucanase in addition to chitinase has been thought to play a major role in biological control of soilborne phytopathogenic fungi by cell wall lysis.

Scanning electron microscopy clearly showed the degradation of *F. solani* mycelium when its cell wall compo-

Table 1. Effect of various carbon sources on β-1,3-glucanase and chitinase production by *P. stutzeri* YPL-1

Carbon source	Enzymatic activity (U)	
	Chitinase	β-1,3-glucanase
Chitin	1.50	–
Colloidal chitin	2.10	–
Laminarin	–	1.32
Laminarin with chitin	1.60	0.10
F. solani mycelium	1.10	0.42

P. stutzeri YPL-1 was grown in a peptone medium containing 0.2% of the various carbon sources. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour. One unit of β-1,3-glucanase activity was defined as the amount of the enzyme releasing 1 μmol of glucose per hour.

Table 2. Antifungal activities of *P. stutzeri* YPL-1 and its mutants against *F. solani*

Mutant	Enzymatic activity (U)		Antifungal activity (%)	
	Chitinase	β-1,3-glucanase	Fungal dry weight[a]	Antagonistic distance[b]
YPL-1	1.65	1.30	100.0	100.0
YPL-M122	0.00	0.00	0.4	0.0
YPL-M153	0.00	0.43	9.0	32.0
YPL-M178	3.50	1.50	156.0	151.5

The mutants were obtained by NTG mutagenesis.
[a] Relative dry weight of *F. solani* with the treatment of the culture filtrates from *P. stutzeri* YPL-1 and its mutant in potato dextrose broth (PDB) after 5 days of incubation at 28℃.
[b] Relative distance between the edges of the bacterial colony and fungal mycelium on potato dextrose agar (PDA) after 5 days incubation at 28℃.

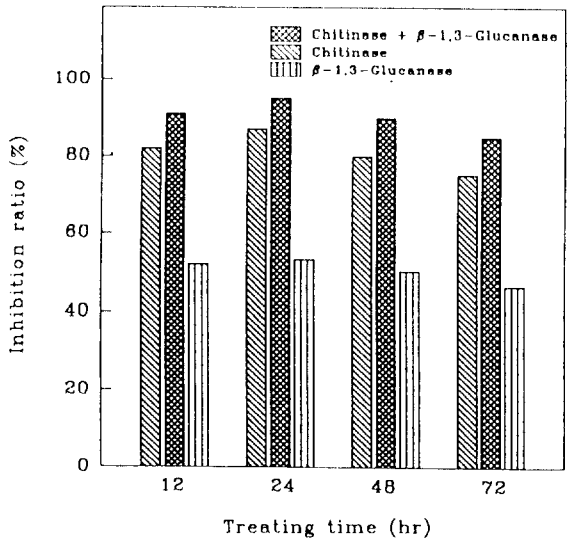

Fig. 1. Antifungal effect of lytic enzymes from *P. stutzeri* YPL-1 on mycelial growth of *F. solani*. The 3-day-old *F. solani* cultures were treated with 10 μg of crude chitinase or 17 μg of crude β-1,3-glucanase from *P. stutzeri* YPL-1. The inhibition ratio was determined by the dry weight of *F. solani* with the treatment of the enzymes relative to a water.

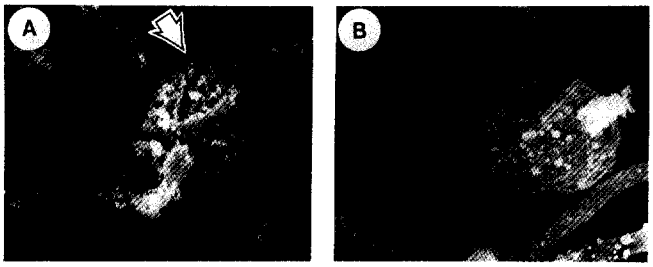

Fig. 2. Scanning electron micrographs of *F. solani* hyphae interacting with *P. stutzeri* YPL-1 in a dual culture. (A) Abnormal hyphal swelling and outflow of cytoplasm (⇨), (B) hyphal retreating and degradation.

nents served as the sole carbon source for *P. stutzeri* YPL-1 in a dual culture (Fig. 2). Abnormal hyphal swelling and retreating were caused by the excretion of the lytic enzymes from *P. stutzeri* YPL-1. It showed that fusarial hyphal walls interacting with accumulation of the bacterial cells were rapidly lysed, causing a leakage of cytoplasm. The use of electron microscopy provided more detailed information about the mode of antagonism and helped to localize sites of interaction between *P. stutzeri* and hyphae of *F. solani*. This result indicates that chitinase and β-1,3-glucanase produced by the antifungal agent *P. stutzeri* YPL-1 attack these sites and completely degrade the hyphae.

In an *in vivo* pot test, *P. stutzeri* YPL-1 proved to have biocontrol ability as a powerful agent in controlling plant disease. Application of kidney bean (*Phaseolus vulgaris* L.) seedling with the bacterial suspension in *F. solani*-infested soil significantly suppressed the development of the root-rot disease after 30 days of treatment (Fig. 3).

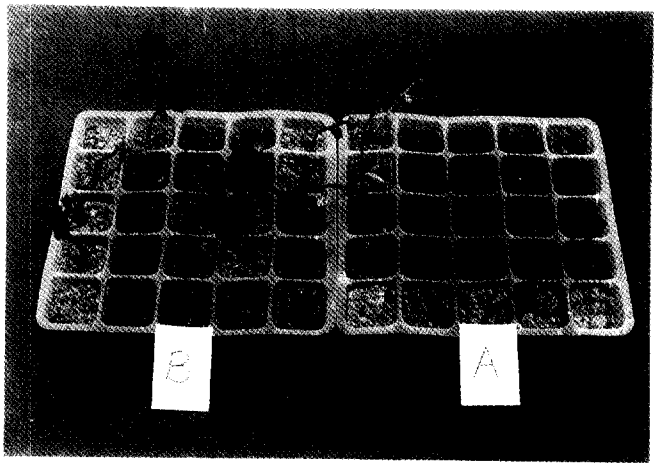

Fig. 3. *In vivo* suppressive effect of *P. stutzeri* YPL-1 against root-rot of kidney bean (*Phaseolus vulgaris* L.) caused by *F. solani*. Polypropylene boxes (50×50×50 mm) were two-third filled with a field soil, and 25 seeds of kidney bean were placed in each box. A seed cover layer (one-third of the pot's depth) was infested with a preparation of *F. solani* (16~20 mg/g soil) and mixed with 5 ml (1×10^8 cells/ml) of *P. stutzeri* YPL-1 suspension. A: *F. solani* only, B: *F. solani* with *P. stutzeri* YPL-1.

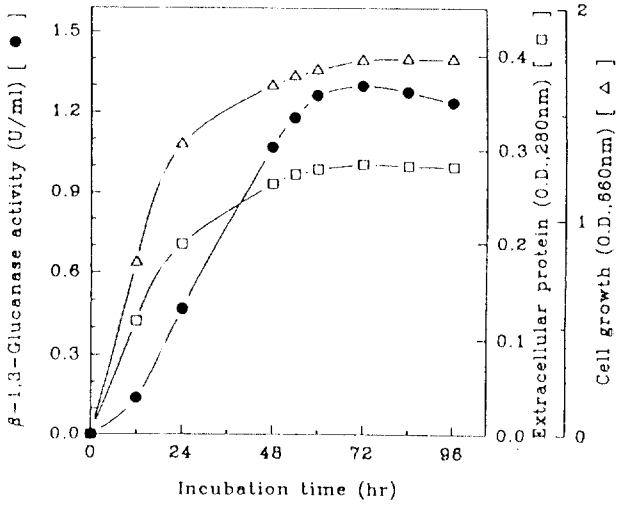

Fig. 4. Growth and β-1,3-glucanase production of *P. stutzeri* YPL-1. *P. stutzeri* YPL-1 was grown at 30°C in a laminarin-peptone medium and the enzyme activity was assayed under the standard conditions.

Growth profile and time course of the β-1,3-glucanase production

The time course of cell growth and β-1,3-glucanase production of *P. stutzeri* YPL-1 are shown in Fig. 4. The cells were cultivated at 30°C in a laminarin-peptone me-

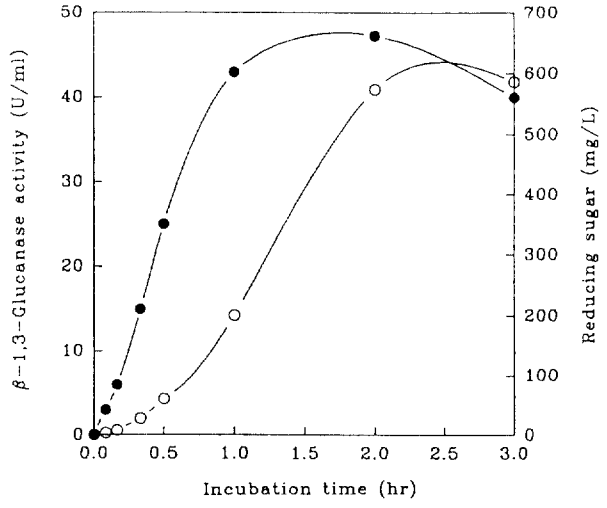

Fig. 5. Time course of reaction hydrolyzed by β-1,3-glucanase. ●: β-1,3-glucanase activity, ○: Amount of glucose released by β-1,3-glucanase.

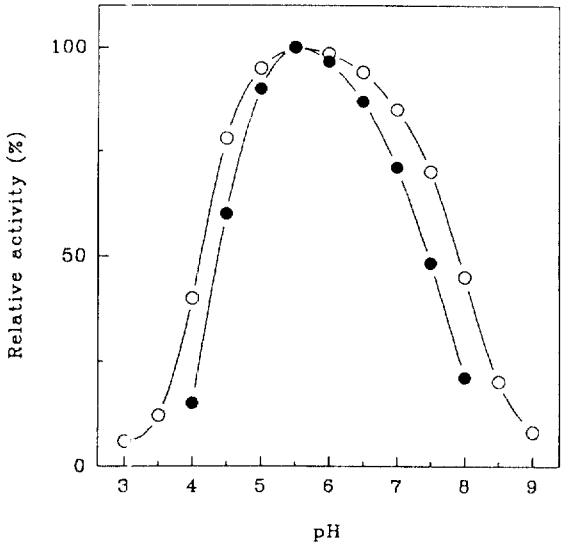

Fig. 6. Effect of pH on the activity and stability of β-1,3-glucanase. The β-1,3-glucanase activity (●) was assayed in a 1 M sodium acetate buffer (pH 4.0~5.5) and a 50 mM sodium phosphate buffer (pH 6.0~8.0) at the various pHs. A β-1,3-glucanase solution preincubated for 30 min in a 1 M sodium acetate buffer (pH 3.0~5.5) and a 50 mM sodium phosphate buffer (pH 6.0~9.0) of various pHs and the remaining activitiy (○) was assayed under the standard conditions.

dium and β-1,3-glucanase production was monitored while the bacterium was growing in the medium. The β-1,3-glucanase activity began when the bacterial growth was in the log phase, and its production increased rapidly after 12 hr of growth. Enzyme production increased in parallel with the growth of the bacterium, and reached its maximum after 72 hr of incubation. Thereafter, the enzyme activity in the culture broth gradually decreased. The decrease in the yield of the enzyme was probably due to the autolysis of the cells and inactivation of β-1,3-glucanase during the death phase.

Hydrolysis of laminarin by β-1,3-glucanase

Time course of the hydrolysis of laminarin by the action of a crude β-1,3-glucanase produced from *P. stutzeri* YPL-1 is shown in Fig. 5. The enzyme hydrolyzed with similar rates in the initial stage of the reaction. However, the amount of reducing sugar released from laminarin reached maximum after 2 hr of incubation, and did not increase during the prolonged incubation.

Effect of pH on activity and stability of β-1,3-glucanase

The effect of pH on the activity of a crude β-1,3-glucanase produced from *P. stutzeri* YPL-1 was determined by varying the pH of the reaction mixtures using a 1 M sodium acetate buffer (pH 4.0~5.5) and a 50 mM sodium phosphate buffer (pH 6.0~8.0). β-1,3-glucanase activity was measured using laminarin as a substrate at 40℃ for 2 hr. The optimal pH range of the enzyme activity was 5.0~6.5 with a maximum activity at pH 5.5 (Fig. 6). The pH optima for β-1,3-glucanase activities were usually in the range of pH 4.5 to 7.5 (2, 4, 19, 22), but exceptions to these were enzymes from *Bacillus brevis* (pH 9.0) with an alkalic optimum pH (15) and *Aspergillus saitoi* (pH 3.8) with an acidic optimum pH (11).

The denaturating effects of pH on β-1,3-glucanase of *P. stutzeri* YPL-1 were investigated by incubating the enzyme solution for 30 min at various pH ranges. The β-1,3-glucanase was preincubated at 40℃ for 30 min at various pHs from 3.0 to 9.0 and the remaining activity was measured using laminarin at pH 5.5. More than 80% of the initial activity of the enzyme was retained at the pH range of 4.5 to 7.0, but was lost dramatically under pH 3.0 and over pH 9.0 (Fig. 6). This result suggests that the β-1,3-glucanase of *P. stutzeri* YPL-1 is more stable at acidic pH than at alkaline pH.

Effect of temperature on activity and stability of β-1,3-glucanase

The effects of temperature on the activity of a crude β-1,3-glucanase produced from *P. stutzeri* YPL-1 were determined by varying the temperature of the reaction mixtures in a 1 M sodium acetate buffer at pH 5.5. β-1,3-glucanase activity was measured using laminarin at pH 5.5 for 2 hr in the temperature range of 20 to 60℃. The enzyme was most active at 40℃ (Fig. 7). The temperature optima for β-1,3-glucanase activities were usually in the temperature range of 40 to 60℃ (4, 11, 22), but exceptions to these were enzymes from *Bacillus circulans* (70℃) or *Bacillus brevis* (65~70℃) with high opti-

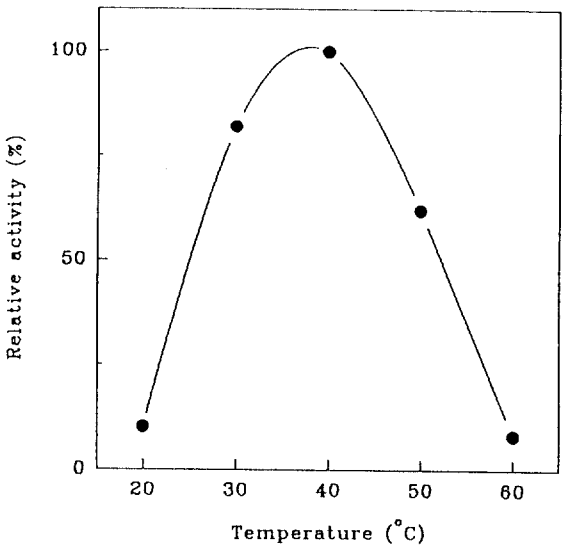

Fig. 7. Effect of reaction temperature on the activity of β-1,3-glucanase. The β-1,3-glucanase activity was assayed in a 1 M sodium acetate buffer of pH 5.5 at various temperatures under the standard conditions.

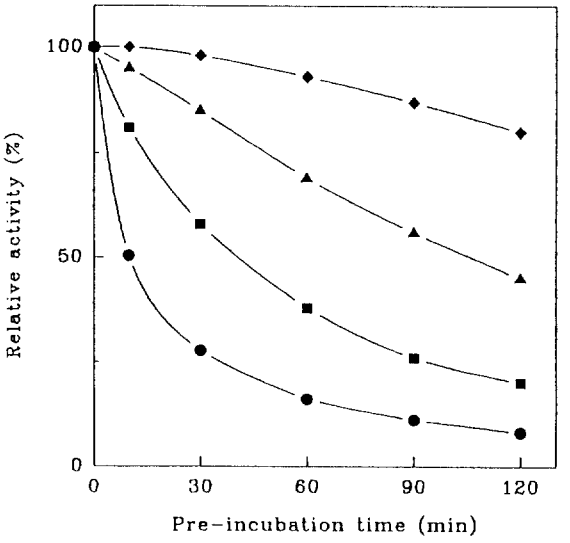

Fig. 8. Effect of temperature on the stability of β-1,3-glucanase. The β-1,3-glucanase solution was preincubated in a 1 M sodium acetate buffer (pH 5.5) at various temperatures from 40℃(◆), 50℃(▲), 60℃(■), and 70℃(●) and the remaining activitiy was assayed under the standard conditions.

mal temperatures (2, 15).

Thermal denaturation of β-1,3-glucanase produced from *P. stutzeri* YPL-1 was examined by maintaining the enzyme solution at different temperatures. The β-1,3-glucanase was preincubated at pH 5.5 at various temperatures from 40 to 70℃, and the remaining activity was measured using laminarin at pH 5.5. The enzyme was quite stable at temperatures below 40℃, lost 40% of the original activity after preincubation for 30 min at 60℃, and was almost inactivated at temperatures above 70℃ for 2 hr (Fig. 8).

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