Mycoscience 35: 169–174, 1994

Fungus fruit body lytic enzyme from a myxomycete, *Badhamia utricularis*

Kazuo Miyairi[1], Kazushige Konno[1], Yukio Harada[2] and Toshikatsu Okuno[1]

[1] Laboratory of Biochemistry and [2] Laboratory of Phytopathology, Faculty of Agriculture, Hirosaki University, Hirosaki, Aomori 036, Japan

Accepted for publication 9 March 1994

Chitinase, β-1,3-glucanase, cellulase, xylanase and protease activity were detected in a crude enzyme preparation obtained from a slime mold (*Badhamia utricularis*) which was grown on autoclaved mycelia of *Pholiota nameko* in a petri dish. The optimal pH of the enzyme preparation for lytic activity against fruit bodies of *Lentinus edodes* was 4.0, and those of β-1,3-glucanase and cellulase were the same. On the other hand, chitinase and protease showed optimal activity at pH 5.0 and 8.0, respectively. The lytic activity was stable below 40°C but completely inactivated at 70°C, and was most stable at pH 5.0. The studies of the optimal pH, thermal stability, and pH stability, and isoelectric focusing analysis of the enzyme preparation suggest that chitinase, β-1,3-glucanase and cellulase activities may be responsible for lysis of fruit bodies of some mushrooms. The crude enzyme preparation from the slime mold lysed fruit bodies of several mushrooms more efficiently than did commercial lytic enzymes preparations (Driselase and Usukizyme).

Key Words——*Badhamia utricularis*; cellulase; chitinase; fruit bodies lysis; β-1,3-glucanase; lytic enzyme; myxomycete.

Introduction

Pholiota nameko (T. Ito) S. Ito et Imai, a familiar edible mushroom in Japan, is sometimes invaded by mycoparasites during its cultivation. One of the authors (Harada, 1977) isolated a myxomycete from rotting fruit bodies of *P. nameko* and identified it as *Badhamia utricularis* (Bull.) Berk. This organism naturally occurs on fruit bodies of many kinds of mushrooms and on fallen trees. The inoculation of the slime mold upon the growing fruit bodies or mycelial of *P nameko* caused their lysis, suggesting that the lytic phenomenon may be due to the action of enzymes produced by the slime mold. The fungus fruit body lytic enzymes from bacteria and fungi have been extensively studied and many papers have reported that the causal enzymes are chitinases and β-1,3-glucanases (Horikoshi, 1963; Toyama et al., 1968). To our knowledge, however, there is no report on lytic enzyme from slime molds. The present study was designed to elucidate the role of enzymes produced by *B. utricularis* in the lysis of fruit bodies of mushrooms and to investigate some properties of those enzymes.

Materials and Methods

Materials Laminarin and carboxymethylcellulose were purchased from Nakarai Chemicals Ltd. and Junsei Chemical Co. Ltd., respectively. Carboxymethylchitin was prepared from chitin by using sodium monochloroacetate (Miyazaki, 1968). Driselase and Usukizyme were products of Kyowa Hakko Kogyo Co. Ltd. and Kyowa Kasei Co. Ltd., respectively. All other chemicals were of analytical reagent grade. Mushrooms used for determining the lytic activity were *Lentinus edudes* (Berk.) Sing., *Pholiota nameko*, *Grifola frondosa* (Dicks.: Fr.) S. F. Gray and *Flammulina velutipes* (Cust.: Fr.) Sing., and they were purchased from the market.

Microorganisms and cultivation The slime mold (*B. utricularis*) isolated from rotting fruit bodies of *P. nameko* in cultivation was grown by the following procedure, principally based on the method of Harada (1977). The swarm cell suspension of *B. utricularis* was sown on living mycelia *P. nameko* in a petri dish (PSA plates were inoculated with sawdust spawn) to form plasmodia. Small portions of plasmodia were then transferred onto autoclaved mycelial colonies of *P. nameko* in a petri dish, and grown for 2 weeks at 20°C after addition of sterilized oatmeal powder as a nutrient. Though slight lytic activities was detected in the crude enzyme extract from mycelia of *P. nameko* in our preliminary experiments, the autoclaved mycelia *P. nameko* was used to deactivate enzymes of mycelia.

Preparation of a crude enzyme mixture from plasmodia of *B. utricularis* The plasmodia on a culture medium (PSA+sawdust) were homogenized with a blender in 40 ml of 0.05 M phosphate buffer (pH 6.0) per petri dish. The homogenized solution was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was dialysed against distilled water at 4°C and lyophilized to give a yellow powder. As a control, a crude enzyme mixture from *P. nameko* mycelia was obtained by the same procedure as that by which the crude enzyme mixture from plasmodia of *B. utricularis* was prepared.

Enzyme assays The activities of chitinase, β-1,3-

glucanase, cellulase, xylanase and mannanase were assayed by using the Somogyi Nelson method (Somogyi, 1952) to determine reducing sugars released from the substrates, namely, carboxymethyl chitin, laminarin, carboxymethyl cellulose, xylane and mannan, respectively. The reaction mixture for the assay consisting of 2 ml of 0.5% substrate solution, 2 ml of 0.25 M acetate buffer (pH 4.0) or 0.1 M phosphate buffer (pH 7.0) and 1 ml of enzyme solution was incubated at 30°C for an appropriate time. A sample (0.5 ml) was taken and 0.5 ml of the alkaline copper reagent was added to terminate the reaction. The amounts of each resultant reducing sugar were estimated from calibration curves prepared for the appropriate monomer. One unit of the each enzyme activity was defined as the amount of enzyme that produced 1 μmole of reducing sugar per min at 30°C. Lytic activity was determined by the same method as described above except that the fruit bodies of *Lentinus edodes* that had been heat treated (100°C, 10 min) to deactivate their enzymes were used as substrate. The reaction mixture consisting of 4 ml of 0.1 M acetate buffer (pH 4.0) or 0.1 M phosphate buffer (pH 7.0) containing fruit bodies of *L. edodes* (3 disks of 7 mm diam) and 1 ml of enzyme solution was incubated on a shaker at 30°C for an appropriate time. The strength of lytic activity was evaluated by estimating the amount of reducing sugar released from a calibration curve of glucose. Lytic activity against cell wall of *Saccharomyces cerevisiae* Hausen (yeast) was also determined by the same method, except for the use of dried yeast as a substrate. Protease activity was assayed by the slightly modified Kunitz method (Oda et al., 1974). The reaction mixture consisting of 0.5 ml of 1.0% casein, 0.5 ml of 0.1 M phosphate buffer pH7.0 and 1.0 ml of enzyme solution was incubated at 30°C for an appropriate time, and then the O.D. at 275 nm was measured. One unit of protease activity was defined as the amount of enzyme which increased the O.D. value by 0.1 per min.

Measurement of protein Protein measured by the method of Lowry (1951) using bovine serum albumin as a standard.

Isoelectric focusing method Isoelectric focusing analysis was carried out by the method of Vesterberg et al. (1966). The crude enzyme preparation (160 mg) was put on a column (110 ml) of carrier ampholytes with a pH range of 3.5 to 10. The electrofocusing was continued for 48 hr at 450 V, and the column was drained and fractionated into 3-ml fractions. Each fraction was tested for pH value and enzyme activities.

Results

1. Enzyme activities of the crude enzyme preparation from plasmodia of *B. utricularis* Various hydrolytic enzyme activities of the crude enzyme preparation were determined. Chitinase, β-1,3-glucanase, cellulase, xylanase and protease activites were detected at the levels shown in Table 1. A lytic activity against fruit bodies of *L. edodes* was also recognized. Mannanase activity and lytic activity against yeast cell walls were only slightly detected. All of these enzymes except protease showed optimal activities at acidic pHs.

2. Enzyme activities of a crude enzyme extract of *P. nameko* A crude enzyme extract from *P. nameko* mycelia was assayed for various enzyme activities. The results are shown also in Table 1. It was found that the enzyme activities in the crude enzyme extract were similar to those from plasmodia of *B. utricularis*. Mannanase activity and lytic activity against yeast cell walls were not detected. Chitinase, β-1,3-glucanase and xylanase showed higher activity at pH 4.0 than at pH 7.0. However, no difference was found in the activity of cellulase between the two pHs.

3. Optimal pHs of the enzymes The optimum pH of lytic activity against fruit bodies of *L. edodes* was found to be 4.0, and those of chitinase, β-1,3-glucanase, and cellulase activities to be 5.0, 4.0 and 4.0 respectively, as shown in Fig. 1. Only protease activity showed a maximum at pH 8.0.

4. pH stabilities of the enzymes The pH stabilities of lytic enzyme, chitinase, β-1,3-glucanase and cellulase were investigated and the results are shown in Fig. 2. Lytic enzyme, chitinase, and β-1,3-glucanase were most stable at pH 5.0, and cellulase at pH 4.0.

5. Thermal stabilities of the enzymes The thermal stabilities of the enzymes are shown in Fig. 3. The lytic en-

Table 1. Enzyme activities in the crude enzyme preparation on from the slime mold and *P. nameko* various substrates.

Enzyme activity	Substrate	*Badhamia utricularis*		*Pholiota nameko*	
		pH 4.0	pH 7.0	pH 4.0	pH 7.0
		(units/petri dish)*			
Chitinase	CM-chitin	28.5	7.5	0.91	0.18
β-1.3-glucanase	Laminarin	10.8	1.9	0.31	0.08
Cellulase	CM-cellulose	9.6	0.2	0.46	0.38
Xylanase	Xylan	7.8	1.1	0.31	0.08
Mannanase	Mannane	0.36	0.27	0	0
Protease	Casein	—	1.6	—	0.94
Lytic activity	*Lentinus edodes*	2.1	0.16	trace	—
	Dry yeast	0.26	trace	—	—

* Protein contents of the crude enzyme preparations from *B. utricularis* and *P. nameko* were 12.5 mg and 46.6 mg per petri dish, respectively.

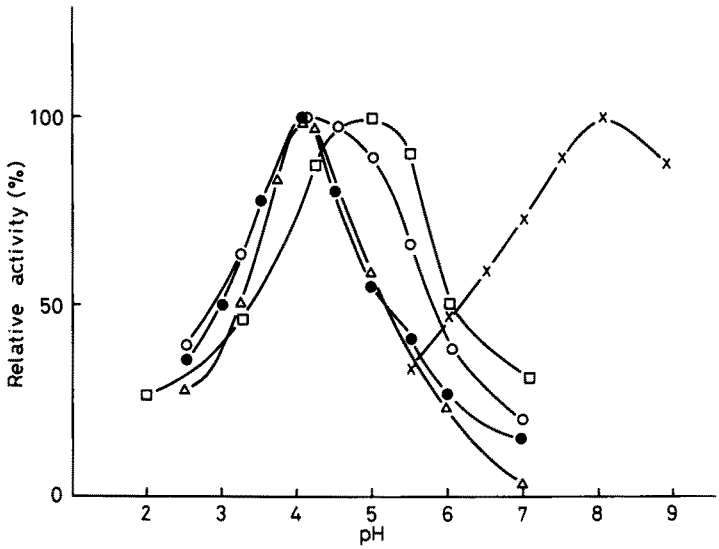

Fig. 1. Effect of pH on various enzyme activities in the crude enzyme preparation from the slime mold.
The enzyme activities were assayed at various pH values under the standard conditions as described in the text. Buffers used were as follows: 0.1 M sodium acetate-HCl buffer (pH 2.0 and 2.5); 0.1 M acetate buffer (pH 3.0 to 5.5); 0.1 M phosphate buffer (pH 6.0 to 7.5); 0.1 M Tris-HCl buffer (pH 8.0 to 9.0). –●–, lytic enzyme; –○–, β-1,3-glucanase; –□–, chitinase; –△–, cellulase; –×–, protease.

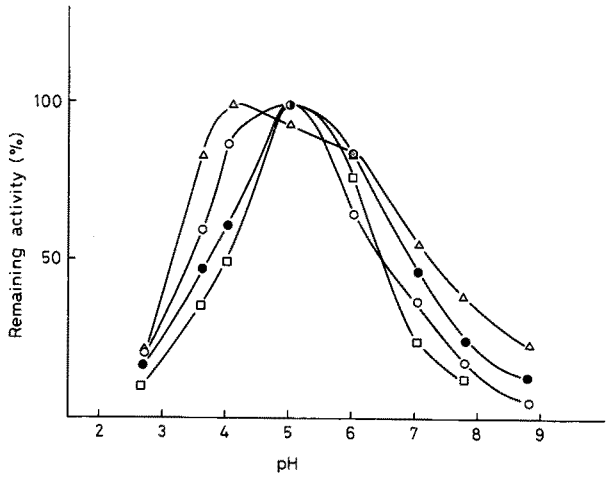

Fig. 2. Effect of pH on stability of various enzymes.
The crude enzyme solution was kept for 10 hr at 30°C in buffers of different pHs (pH 2.6 to 8.8) and the remaining activities were assayed under the standard conditions as described in the text. Buffers used were the same as in Fig. 1. –●–, lytic enzyme; –○–, β-1,3-glucanase; –□–, chitinase; –△–, cellulase.

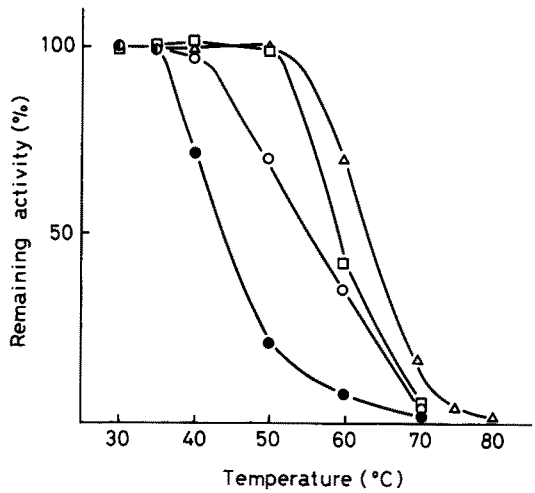

Fig. 3. Effect of temperature on stability of various enzymes.
The crude enzyme solution in 0.1 M acetate buffer (pH 5.0) was kept for 15 min at various temperatures as indicated and the remaining activities were assayed under the standard conditions as described in the text. –●–, lytic enzyme; –○–, β-1,3-glucanase; –□–, chitinase; –△–, cellulase.

zyme activity was very sensitive to a temperature above 40°C. After incubation at 50°C, only 20% of the original activity was retained. On the other hand, chitinase and cellulase were fairly stable up to 50°C, and about 42% and 70% of the original activities were retained at 60°C, respectively. The β-1,3-glucanase also held 80% of the original activity at 50°C. All enzymes other than cellulase, which lost its activity at 80°C, were inactivated completely at 70°C.

6. Effects of various metal ions or compounds on the lytic activity Table 2 summarizes the effects of various metal ions or compounds on the lytic activity. No stimulating effects were observed at the concentration of 1 mM of each metal ion. Mercuric ion completely inhibited the enzyme activity. Other metal ions inhibited 24 to 78% of the original activity at the concentration of 1 mM. Both SH reagents inhibited about 30%.

7. Analysis of the enzyme preparation by isoelectric

Table 2. Effects of metal ions or compounds on the lytic activity.
Lytic activity was measured in the presence of various metal ions or compounds under the standard conditions as described in the text.

Materials	Final concentration (mM)	Relative activity (%)
None	1	100
$CuCl_2$	1	45
$ZnSO_4$	1	47
$SnCl_2$	1	76
$SrCl_2$	1	22
$HgCl_2$	1	0
Iodoacetamide	1	71
PCMB*	1	72
EDTA	1	99

* Sodium *p*-chloromercuribenzoate.

focusing method Isoelectric focusing analysis over a wide pH range (pH 3.5–10) was carried out to separate enzymes assumed to be responsible for the lysis of fruit bodies from the others. Elution patterns of the chitinase, β-1,3-glucanase, cellulase, protease and lytic enzyme in isoelectric focusing are illustrated in Fig. 4. Lytic activity was observed as two peaks, a large one at pH 3.9 and a small one at pH 5.3. The major peak of lytic activity overlapped one peak of the cellulase and partially overlapped those of the chitinase and β-1,3-glucanase.

8. Lysis of several mushrooms by the enzyme preparation Finely cut fruit bodies of *L. edodes, P. nameko, G. frondosa* and *F. velutipes* were treated with the enzyme preparation and amounts of reducing sugars released from each fruit body were measured. Their time-courses are summarized in Fig. 5. The fruit bodies of *F. velutipes* were decomposed at a fast rate, but those of *G. frondosa* most slowly, showing only 25% of the former even after 160 min. A characteristic of the enzyme action, except on *G. frondosa*, was that the rates of decomposition were accelerated with the lapse of time. After a week, all fruit body tissues of mushrooms used appeared to the naked eye to be completely degraded.

9. Comparisons of lytic patterns of fruit bodies of *L. edodes* by the slime mold lytic enzyme with those by commercial ones The lytic activity of the slime mold enzyme against fruit bodies of *L. edodes* was compared with those of commercial lytic enzymes (Driselase and Usukizyme) (Fig. 6). Though there were no differences in hydrolytic rates among the tested enzyme preparations during the initial 30 min of reaction, the enzyme preparation from *B. utricularis* showed the highest activity after 3 h. After four days, degrees of fruit body degradation with *B. utricularis* enzyme, Usukizyme and Driselase reached 82.2% 57.6% and 64.5%, respectively.

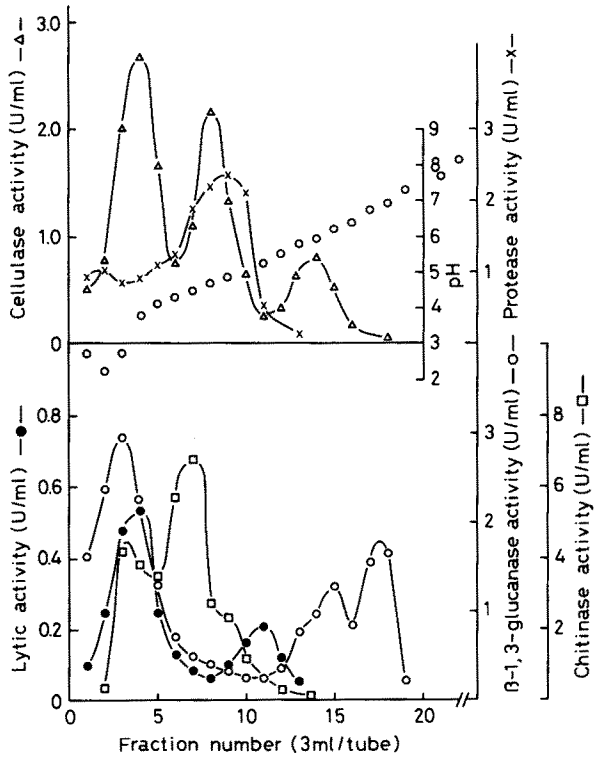

Fig. 4. Isoelectric focusing of the crude enzyme preparation from the slime mold.
Conditions are detailed in the text. The electrofocusing was done at 4°C for 48 h by using carrier ampholine in a sucrose density gradient. Fractions of 3 ml were collected and measured for pH values and enzyme activities. ○, pH; —●—, lytic enzyme; —○—, β-1,3-glucanase; —□—, chitinase; —△—, cellulase; —×—, protease.

Discussion

The crude enzyme mixture produced by *B. utricularis* was found in our preliminary experiment to lyse fruit bodies of *L. edodes* as well as *P. nameko*. To determine the lytic activity, therefore, cut fruit bodies of *L. edodes* having their enzymes deactivated by heating were used as a substrate, because *L. edodes* had less mucilage than *P. nameko* on the pileus. The lytic activity was assayed by determination of reducing sugars produced, because the

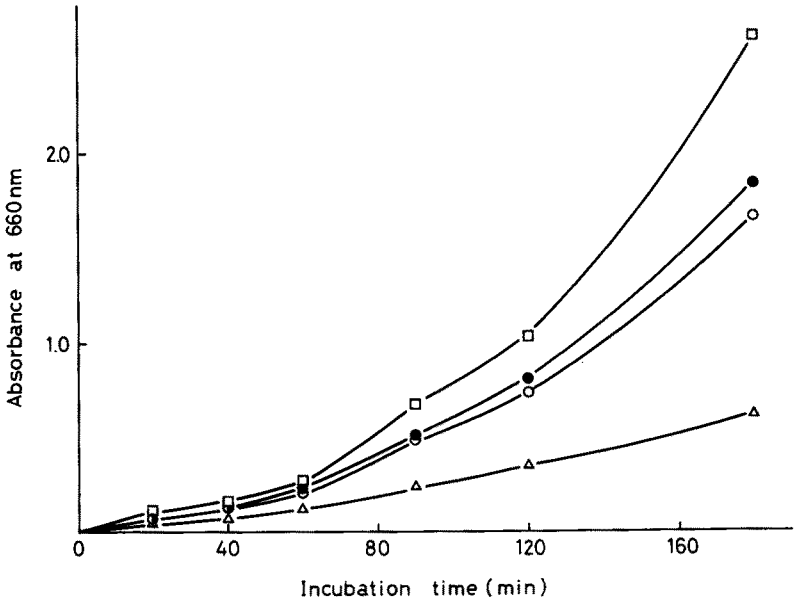

Fig. 5. Lysis of several mushrooms with the crude enzyme preparation from the slime mold.
The reaction mixture, consisting of 5 ml of 0.1 M acetate buffer (pH 5.0) containing 300 mg (fresh weight) of each mushroom, 0.25 unit of the crude enzyme as a β-1,3-glucanase, and 0.02% sodium azide as a preservative was incubated on a shaker at 30°C. The reducing sugar produced were determined colorimetrically according to the Somogyi Nelson method. –●–, *L. edodes*; –○–, *P. nameko*; –△–, *G. frondosa*; –□–, *F. velutipes*.

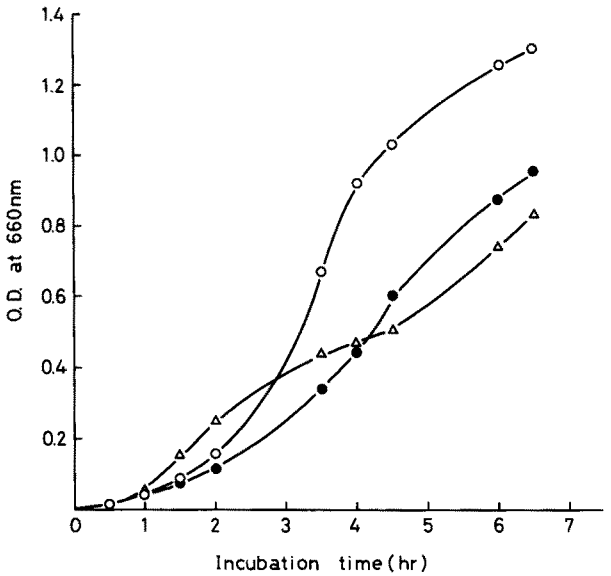

Fig. 6. Comparison of lytic patterns of the fruit bodies of *L. edodes* by the slime mold lytic enzyme with those by commercial ones.
The reaction solution, 3 ml of 0.1 M acetate buffer (pH 4.5) containing fruit bodies of *L. edodes* (dry wt. 200 mg), was incubated on shaker at 30°C after adding each enzyme preparation (0.25 unit as a β-1,3-glucanase). The reducing sugar formed was determined colorimetrically according to the Somogyi Nelson method. –○–, slime mold lytic enzyme; –●–, Usukizyme; –△–, Driselase.

reproducibility of the data of turbidity changes resulting from fruit body digestion was poor.

The action ranges of pH of the component enzymes of the crude enzyme preparation, except for the protease, were acidic (pH 2.5–6.0) (Fig. 1). No evidence was found for the presence of acid protease activity even when hemoglobin, which was known to be a better substrate in that assay, was used instead of casein. The optimal pHs of cell wall lytic enzymes from fungi so far reported (Toyama et al., 1968) were at around 5.0, and those values are nearly equal to those of the slime mold (pH 4.0–5.0).

In pH stability experiments, the chitinase, β-1,3-glucanase and cellulase activities were stable at pH 4.5–5.5, and the range of stability was similar to that of lytic enzyme (Fig. 2). The lytic enzyme was more unstable to heating than the other three enzymes, which differed from one another in thermal stability. These results suggest that the lysis of fruit bodies was caused by the combined actions of more than one enzyme activity.

In isoelectric focusing analysis, the lytic activity of the enzyme preparation from the slime mold was assumed to be very complex, as each enzyme activity was composed of several isozymes (Fig. 4). The peaks of chitinase, β-1,3-glucanase and cellulase activities overlapped the major peak of lytic activity, and the peak of lytic activity was in good agreement with one peak of cellulase activity.

From the above results, it seems that the chitinase, β-1,3-glucanase and cellulase activities of this slime mold are responsible for lysis of fruit bodies of *L. edodes* and *P.*

nameko. In previous studies, chitinase and β-1,3-glucanase have been suggested to be casual enzymes of lysis of fungus cell walls (Toyama et al., 1968; Peberdy, 1988), but the participation of cellulase in lysis has not been mentioned. Although it is not clear whether or not cellulose or a similar component as a substrate of cellulase are present in fruit bodies of these two mushrooms, Marchant (1966) reported the presence of a small amount of cellulose in both conidial and hyphal walls of *Fusarium culmorum*. Also, cellulase has been used together with other enzymes for preparing protoplasts from basidiomycete mycelia (Ushiyama et al., 1977; Ohmasa et al., 1987). These facts suggest that in *L. edodes* and *P. nameko*, there may be glucan that is lysed by cellulase. But further studies are needed to clarify the role of cellulase.

The lytic rates of the enzyme preparation for four kinds of mushrooms used were fairly different; the rate for *F. velutipes*, a soft mushroom, was fastest, while that for *G. frondosa*, a hard one, was slowest. Generally, the lytic rates for all mushrooms accelerated with the progress of the reaction (Fig. 5). This phenomenon seemed to be caused by the difference in cell wall structures between the outer and inner layers of fruit bodies. Wessels et al. (1972) studied the cell wall polysaccharides of *Schizophyllum commune* Fr.: Fr., and reported that α-1,3-glucan was present in the outer layer of cell wall, while highly branched glucan was present in the inner one, in which chitin microfibrils were embedded. Since *L. edodes* and *P. nameko* as well as *S. commune* are basidiomycetes, it may be reasonable to assume that they have cell walls of a similar heterogeneous structure.

Compared with the commercial lytic enzymes, Usukizyme and Driselase, the slime mold enzyme proved to be more active against lysis of fruit bodies of mushrooms (Fig. 6). Therefore, the slime mold lytic enzyme may serve as a useful agent in lysis of fruit bodies of mushrooms. Whether or not it is effective for lysis of bacteria and filamentous fungi remains to be clarified.

Although there have been many reports on lytic enzymes obtained from bacteria (Horikoshi et al., 1963) and fungi (Tokimoto, 1982; Kitamoto et al., 1987), to our knowledge this is the first report on lytic enzyme from a slime mold. It may become a subject of considerable interest.

Literature cited

Harada, Y. 1977. *Badhamia utricularis* occurring on fruit bodies of *Pholiota nameko* in sawdust culture. Bull. Fac. Agric. Hirosaki Univ. **28**: 32–42.

Horikoshi, K., Koffler, H. and Arima, K. 1963. Purification and properties of β-1,3-glucanase from the "Lytic enzyme" of *Bacillus circulance*. Biochim. Biophys. Acta **73**: 268–275.

Kitamoto, Y., Kagawa, I., Nagao, N. and Nakamata, M. 1987. High-productivity protoplasting and reversion of protoplasts in *Coprinus cinereus* with a single preparation of lytic enzyme from *Trichoderma harzianum*. Trans. Mycol. Soc. Japan **28**: 217–227.

Lowry, O., Rosenbrogh, O. H., Farr, N. J. and Randoll, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.

Marchant, R. 1966, Wall structure and spore germination in *Fusarium culmorum*. Ann. Bot. **30**: 821–830.

Miyazaki, T. and Matsushima, Y. 1968. Studies on Aminohexoses XIV. Bull. Chem. Soc. Japan **41**: 2723–2726.

Oda, K. and Murao, S. 1974. Purification and some enzymatical properties of acid protease A and B of *Scytalidium lignicolum* ATCC 24568. Agric. Biol. Chem. **38**: 2435–2444.

Ohmasa, M., Abe, Y., Furukana, H., Taniguchi, M. and Neda, H. 1987. Preparation and culture of protoplasts of some Japanese cultivated mushrooms. Bull. Forest. Forestry Products Research Institute **343**: 55–170.

Peberdy, J. F. 1989. Presidential address: Fungi without coats—protoplasts as tools for mycological research. Mycol. Res. **93**: 1–20.

Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. **195**: 19–23.

Toyama, N. and Ogawa, K. 1968. Purification and properties of *Trichoderma viride* Mycolytic enzyme. J. Ferment. Technol. **46**: 626–633 (in Japanese).

Ushiyama, R. and Nakai, Y. 1977. Protoplasts of Shiitake, *Lentinus edodes* (Berk.) Sing. Rept. Tottori Mycol. Inst. **15**: 1–5 (in Japanese).

Vesterberg, O. and Svenson, H. 1966. Isoelectric fractionation analysis and characterization of ampholytes in natural pH gradation. Acta Chem. Scand. **20**: 820–834.

Wessels, J. G. H., Krreger, D. R., Marchant, R., Regensburg, B. A. and de Vries, O. M. H. 1972. Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. Biochim. Biophys. Acta **273**: 346–358.