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Studies on *Ganoderma lucidum*
III. Production of pectolytic enzymes

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Abstract. Pectolytic enzymes produced by *Ganoderma lucidum* B in culture and polypropylene bags were investigated. Two pectolytic enzymes, *e.g.*, endo-polygalacturonase (endo-PG) and endo-pectin methyl *trans*-eliminase (endo-PMTE) were obtained from crude extract of mycelia cultured in polypropylene bags. The endo-PMTE has two optimal pH at 4.5 and 8.0. The enzyme was stimulated by Ca^{2+} and preferred only pectin as substrate. The enzyme activity decreased at temperature above 50°C. The endo-PMTE a and endo-PMTE b, obtained from mycelia of *G. lucidum* B in polypropylene bags, were purified by 60-80% ammonium sulfate fractionation, Sephadex G-100 gel filtration, DEAE-cellulose ion exchange column chromatography and isoelectric focusing, showing pI at 8.2 and 5.5. Disc gel electrophoresis confirmed two peaks corresponding to endo-PMTE a and b as isoenzymes. The crude endo-pectin methyl *trans*-eliminases were finally purified about 10 folds. Pectolytic enzymes purified by 60-80% ammonium sulfate fractionation macerated potato disc more actively than the crude enzymes. At pH 4.5, maceration of potato disc by pectolytic enzymes was more effective than those at pH 8.0 or 7.0. At pH 8.0, Ca^{2+} stimulated pectolytic enzyme activities and accelerated maceration. This is an original report revealed that *G. lucidum* produced endo-PMTE.

Key words: endo-pectin methyl *trans*-eliminase; endo-polygalacturonase; *Ganoderma lucidum*; maceration.

Introduction

Ganoderma lucidum is a wood rotting fungus and also known as a traditional Chinese medicine. Although the pathogenicity of the fungus has been well documented (Venkataryan, 1936; Naidu, *et al.*, 1966; Navuratnam and Leong, 1965; Mahmood, 1971), little is known about the biochemical and physiological aspects of this disease.

In many plant diseases the pectolytic enzymes of pathogens have been most widely studied in relation to soft rot induction. Polygalacturonase and other macerating enzymes for instance play an important role in pathogenesis (Bateman and Millar, 1966; Stephens and Wood, 1975; Tseng and Tseng, 1980; Forster and Rasched, 1985). Such enzymes may be responsible for formation of rot induced by *G. lucidum*. However, no reports on pectolytic enzymes of this species were found in the literature, except one isolate of *G. lucidum* from India indicated to produce an endopolygalacturonase *in vitro* (Kumari and Sirsi, 1971). In our previous reports the chemical compositions of *G. lucidum* and the effects of the fungus on lipid

metabolism in rat were elucidated (Tseng *et al.*, 1984; Shaio *et al.*, 1986). This paper deals with the production of an endo-pectin methyl *trans*-eliminase and endo-polygalacturonase by *G. lucidum*.

Materials and Methods

Culture and Enzyme Sources

An isolate of *Ganoderma lucidum* B was kindly obtained from Mr. P.C. Chang, Department of Plant Pathology, National Taiwan University. The culture was maintained on potato dextrose agar at 28±2°C and transfers were made at 2-week intervals. Crude enzyme preparations were obtained from various ways. *Ganoderma lucidum* was grown in 200 ml of MD broth (3% of malt extract; 2% of dextrose) in a 500 ml Erlenmeyer flask at 28±2°C for 14 days. The mycelia were harvested with 4 layers of cheesecloth, and the filtrates were ready for enzyme assays. The mycelial cakes were cut into small pieces, suspended in buffer solution at a ratio of 1 g mycelium to 5 ml of 50 mM phosphate buffer (pH 6.5) and homogenizing by a Virtis '45' at high speed in the cold for 1 min. The homogenates were centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was lyophilized and saved at −20°C until used. In order to cultivate artificial fruiting bodies, *G. lucidum* was grown in sterilized polypropylene bags which contained 400 g of acacia chip, 80 g of rice bran, 12 g of sucrose, 0.4 g of $(NH_4)_2SO_4$, 0.4 g of peptone and 2 g of $Ca(SO_4)_2 \cdot 2H_2O$ to make up a 50% water content. Bags were incubated at 28±2°C for three weeks and then transferred to a green house. Sixty days after inoculation, the fruiting bodies of the fungus developed on almost all of the bags. The artificial fruiting bodies and its mycelia in polypropylene bags were collected separately, homogenated and lyophilized as previously described. The lyophilized materials were served as sources of crude enzymes.

Enzyme Assay

Pectolytic enzymes were initially measured by cup-plate method as described by Dingle *et al.* (1963), using 1.2% each of pectin N.F. (Calbiochem product, Los Angeles 63, USA) and sodium polypectate (Nutritional Biochemical Cooperation, Cleveland, Ohio, USA) as substrates with 50 mM of citrate buffer at pH 4.5 and Tris-HCl buffer at pH 8.0. It was demonstrated that the diameter of the zone of pectin hydrolysis in cup plates was proportional to the enzyme concentrations between 1 and 6 mg protein per milliliter, if crude lyophilized enzyme of mycelium from polypropylene bag was used. The assay medium contained 1.2% pectin, 2% agar, 0.01% thiomerealate in 50 mM of Tris-HCl buffer at pH 8.0. Diameters of the zones of hydrolysis were measured after 24 h incubation at 30°C (Fig. 1). The enzyme activities were also assayed by viscometric method (Bateman, 1966).

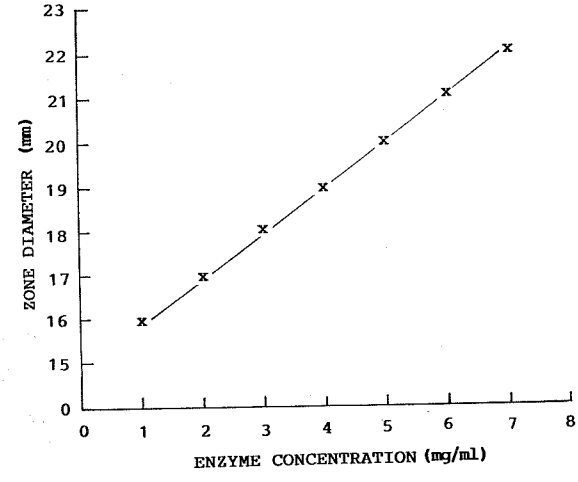

Fig. 1. The relationship of the zone diameter of pectin hydrolysis and *trans*-cleavage by pectolytic enzyme of *Ganoderma lucidum* B in the cup-plate assay. The enzyme source for this experiment was crude lyophilized extract from mycelia in polypropylene bags. The assay medium contained 1.2% pectin, 2% agar, 0.01% thiomersalate, and 50 mM Tris-HCl buffer (pH 8.0). Wells made in a 5-mm layer of the solidified substrate, 9 mm in diameter, and contained 0.2 ml of a given enzyme concentration. Diameters of the zones were measured after 24 h incubation at 30°C.

Reaction mixture contained 4 ml of 0.6% sodium polypectate or pectin in 50 mM of various buffers at different pH values (citrate buffer, pH 3.0-5.5; phosphate buffer, pH 6.0-7.0 and Tris-HCl buffer, pH 7.5-9.0) plus 2 ml of distilled water and 1 ml enzyme preparation. It was incubated in 30°C water bath. The enzyme activity was expressed in relative activity units ($1/t \times 10^3$, where t is the time in minutes for 50% viscosity loss).

Polygalacturonate *trans*-eliminase (PGTE) and pectin methyl *trans*-eliminase (PMTE) were measured by the periodatethiobarbituric acid (TBA) method (Presis and Ashwell, 1963) at 30°C. The reaction mixture contained 0.1 ml of 0.6% sodium polypectate or pectin in 50 mM Tris-HCl buffer (pH 8.0) plus 10^{-3} M $CaCl_2$ and 0.1 ml enzyme preparation at 30°C for 1 h. One unit of PGTE or PMTE activity was expressed as the amount of enzyme giving an increase in absorbance of 0.1 in 60 min at 548 nm wavelength in TBA assay. Autoclaved enzymes were served as controls and protein content was measured by Lowry method (1951).

Enzyme Purification

Crude enzyme (100 ml) from mycelia polypropylene bags was brought to 20% saturation with ammonium sulfate, allowed to stand for 1 h at 4°C, and centrifuged for 20 min at 20,000 xg. The precipitate was collected and suspended in 15 ml of distilled water. It was repeated for ammonium sulfate saturation to 40, 60, 80 and 95%. The precipitate of each fraction was assayed for pectolytic enzyme activity.

In the preliminary study, a bulk of endo-PMTE activity was located in the 60-80% $(NH_4)_2SO_4$ fraction. This fraction was then applied to a 2.6×70 cm column of Sephadex G-100 equilibrated with 50 mM Tris-HCl buffer at pH 8.0. The column was eluted with the same buffer at 4°C. Five ml fractions were collected and aliquot of the fractions was assayed for the enzyme activity. The fractions which contained endo-PMTE were pooled and applied to a 1.6×30 cm column of diethylaminoethyl cellulose (Cl^- form, DEAE cellulose) equilibrated with 50 mM Tris-HCl buffer at pH 8.0. The column was eluted with 30 ml of buffer followed by a NaCl gradient in buffer until 0.2 N was reached. Five ml fractions were collected and assayed for the enzyme activity.

The endo-pectin methyl *trans*-eliminase peak fractions from the DEAE cellulose column were combined and dialyzed for 24 h against distilled water. This enzyme fraction was subjected to isoelectric focusing in a LKB 8101 Ampholine electrofocusing apparatus as previously described (Tseng and Tseng, 1980). Purified enzyme was further analyzed by disc gel electrophoresis (Hedrick and Smith, 1968).

Measurement of Tissue maceration by ***Ganoderma lucidum*** *Pectolytic Enzymes*

Tissue maceration was evaluated and rated as described by Mount *et al.*, (1970). Cylinders of fully turgid potato tuber were cut with a corkborer (9 mm in diameter.), and discs (1 mm thick) were cut with a hand microtome and washed in distilled water. The washed discs were placed in reaction mixture (6 discs/ml) containing equal volume of buffers (50 mM citrate buffer pH 4.5; 0.05 M phosphate buffer pH 7.0; 50 mM Tris-HCl buffer pH 8.0) and enzymes at 28°C. Tissue maceration was made over 9-h incubation period. Discs which were not macerated received a rating of 0, and those completely macerated received a score of 5.

Results

Based on cup-plate assay, Fig. 2 illustrates that no pectic glycosidases and *trans*-eliminases activities were detected either from the culture filtrates and mycelial cakes or fruiting bodies of *G. lucidum*, at pH 4.5 and 8.0 with pectin or sodium polypectate as substrate. However, polygalacturonase and pectin methyl *trans*-eliminase were speculated in crude enzyme extracts from mycelia polypropylene bags. There were further confirmed

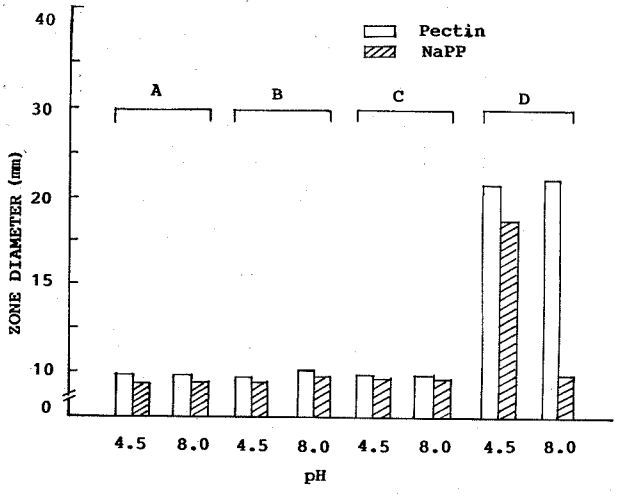

Fig. 2. Pectolytic enzyme activities of crude enzyme preparations of *Ganoderma lucidum* B. Enzyme sources: culture filtrate (A); mycelial cake (B); fruiting body (C); crude enzyme extract from mycelia in polypropylene bag (D). The enzyme activities were assayed by cup-plate method.

by viscometric and TBA assays. As shown in Table 1, an enzyme, which exhibited a high catalytic activity at pH 4.5 with sodium polypectate as substrate and was inhibited by 1 mM $CaCl_2$, rapidly caused over 50% viscosity loss in ten minutes and failed to show the enzyme activity by TBA assay was classified as an endo-polygalacturonase. A pectin methyl *trans*-eliminase was also detected in the enzyme preparations both at pH 4.5 and 8.0 preferred pectin as substrate. The enzyme activity, which could be stimulated by 1 mM $CaCl_2$, caused a rapid loss of viscosity in 10 min on its substrate but released less amount of reducing sugars in reaction mixture assay by Nelson test (1944), was also observed and characterized as an endo-PMTE. No endo-PGTE was detected in the enzyme preparation. The optimal pH of the crude pectolytic enzymes was further studied at a broad pH range. Results showed three optimal pH at 4.5, 6.5 and 8.0 (Fig. 3). The thermostability of the enzymes was examined at pH 4.5 and 8.0. There was no significant loss of its activity at 10°C up to 40°C as compared with the control. However, the enzyme activity decreased at temperature above 50°C (Fig. 4).

Purification of ***Ganoderma lucidum*** *B Endo-pectin Methyl* ***trans****-eliminase*

One hundred milliliters of crude enzyme was dialyzed against distilled water at 4°C for 24 h. The enzyme contained 2.61 mg protein/ml and had a specific activity of 1.94.

The dialyzed enzyme was subjected to $(NH_4)_2SO_4$ fractionation. The supernatant obtained after 80% saturation, which contained most of the enzyme activity (specific activity=3.17), was dialyzed and used for the next step purification by a Sephadex G-100 column. Results indicated that only one endo-PMTE activity peak (No. 54-59) was collected in the column (Fig. 5). The specificity activity of this partly purified enzyme was 6.46. Further purification of the enzyme was achieved by a DEAE cellulose column with a NaCl gradient in buffer. As shown in Fig. 6, the total endo-PMTE activity was located in fractions No.

Table 1. *Pectolytic enzyme activities of* ***Ganoderma lucidum*** *B with pectin and sodium polypectate (NaPP) as substrates*

Assay	pH 4.5		pH 4.5 (1 mM $CaCl_2$)		pH 8.0		pH 8.0 (1 mM $CaCl_2$)	
	Pectin	NaPP	Pectin	NaPP	Pectin	NaPP	Pectin	NaPP
Viscometric method	185[1]	125	192	59	164	0	172	0
TBA method	0.75[2]	0	0.93	0	2.10	0	2.89	0

[1] Relative activity unit=$1/t \times 10^3$, where t is the time in minutes for 50% viscosity loss.
[2] Specific activity=unit/mg protein.

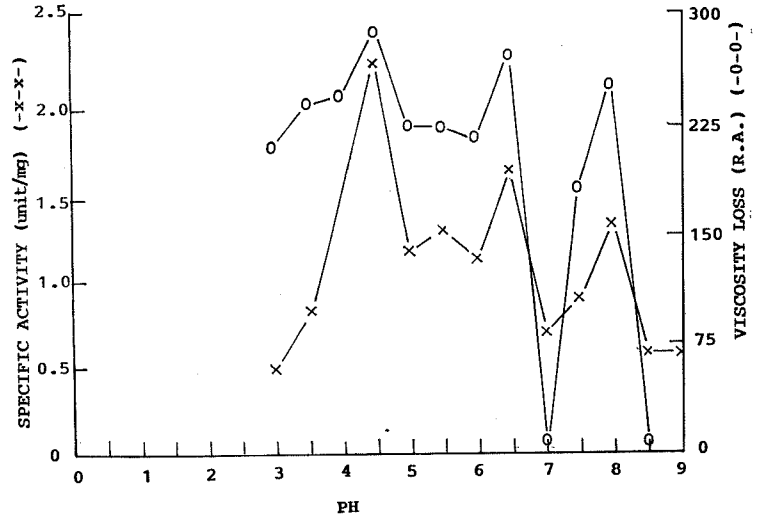

Fig. 3. Effect of pH on crude pectolytic enzymes of *Ganoderma lucidum* B. Enzyme activity was estimated by the methods of TBA (-×-×-) and viscometric assay(-0-0-).

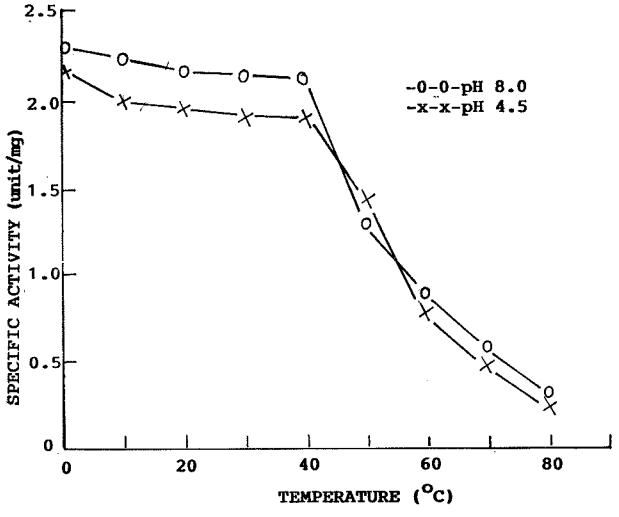

Fig. 4. Effect of temperature on the activities of crude pectolytic enzymes of *Ganoderma lucidum* B. The enzyme was treated at the indicated temperature for 60 min and assayed by TBA method.

9-11 (specific activity=18.33). The partially purified enzyme was pooled, dialyzed and finally subjected to isoelectric focusing by using a Ampholine carrier with a pH range of 3.5-10. Figure 7 shows the enzyme activity peak consists of two not completely separated peaks, suggesting that the purified endo-PMTE from DEAE cellulose column composed of two different isoenzymes. These are designated as endo-PMTE a and endo-PMTE b which showed pI values of 8.2 and 5.5, respectively. The partially purified endo-PMTE from chromatofocusing column were further subjected to disc gel electrophoresis for evaluating its homogeneity. Results confirmed two peaks correlating to endo-PMTE a and b as isoenzymes (Fig. 8). The molecular weights of the isoenzymes were not determinated in the current study. The crude endo-PMTE was finally purified about 10-fold. The summary of the purification of *G. lucidum* endo-PMTE was illustrated in Table 2.

Tissue Maceration by Pectolytic Enzymes Produced by ***G. lucidum*** *B*

Table 3 shows that the loss in coherence of discs from potato tuber treated with crude enzyme preparation and the ammonium sulfate fractionation of the cultural extract were no identical. Apparently, pectolytic enzymes of 60-80% ammonium sulfate fractionation macerated potato discs more actively than the crude enzymes. At pH 4.5 and 8.0, maceration of potato discs by the pectolytic enzymes were more effective than

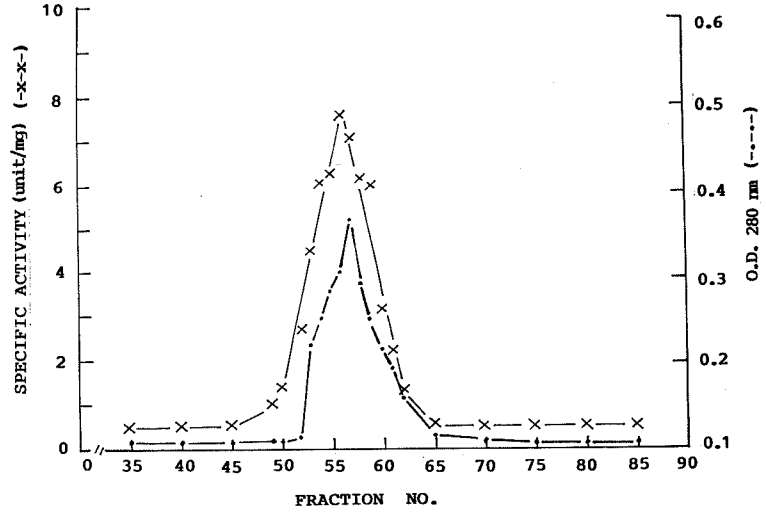

Fig. 5. Elution profile of endo-pectin methyl *trans*-eliminase (endo-PMTE), in an ammonium sulfate fraction (60-80%) of crude enzyme of *Ganoderma lucidum* B, by Sephadex G-100 column chromatography. Fifteen ml of the enzyme was applied to a 2.5×70.0 cm column. Five-ml fractions were collected and the enzyme activity was determined by TBA method.

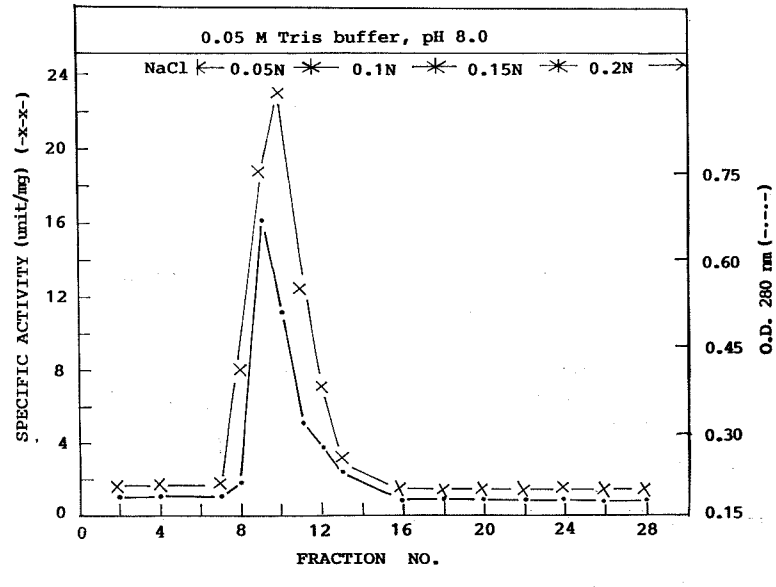

Fig. 6. Purification of endo-pectin methyl *trans*-eliminase partially purified from gel filtration column (Fig. 5. fraction No. 54-59) by DEAE cellulose column chromatography. Thirty ml of the enzyme was applied to a 1.6×30.0 cm column, using NaCl as salt gradient. Five-ml fractions were collected and the enzyme was assayed by TBA method.

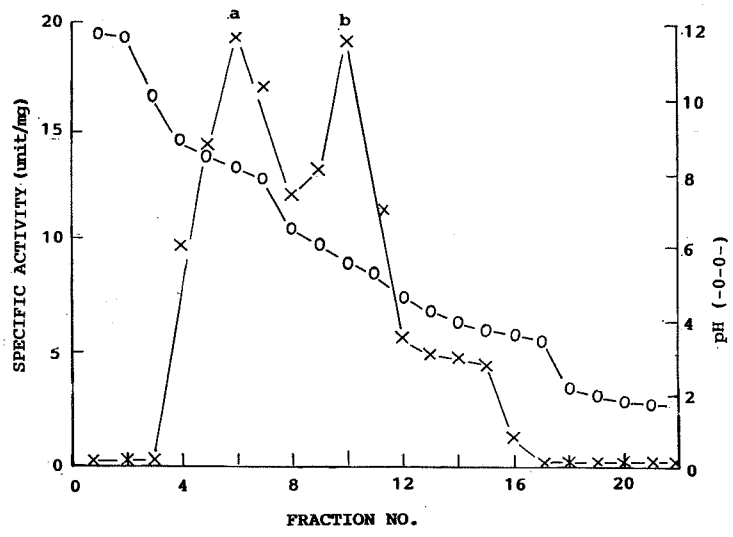

Fig. 7. Isoelectric focusing of dialyzed endo-pectin methyl *trans*-eliminase from DEAE cellulose column (Fig. 6, fractions No. 9-11, 15 ml). A pH range of 3.5-10 Ampholine carrier was used. Five-ml fractions were collected and the enzyme was assayed by TBA method.

those at pH 7.0. At pH 8.0, Ca^{2+} stimulated pectolytic enzyme activities and accelerated maceration. These results indicated multiple endo-PMTE and endo-PG existed in 60-80% ammonium sulfate fractionation.

Discussion

Pectolytic enzymes produced by *G. lucidum* B in liquid culture without pectic substances and polypropylene bags were studied. Fungus inoculated in polypropylene bags contains higher pectolytic enzyme activities than those from cultural filtrates, mycelial cakes and fruiting bodies. These results suggest that pectolytic enzymes in *G. lucidum* are inducibe enzymes. Based on viscometric and TBA assays, we found crude pectolytic enzymes of *G. lucidum* B extracts from mycelia polypropylene bags have three optimal pH of 4.5, 6.5 and 8.0 (Fig. 3). A crude enzyme at pH 4.5 preferred sodium polypectate as substrate, inhibited its enzyme activity by Ca^{2+} was defined as an endo-polygalacturonase (endo-PG). At pH 8.0, a crude enzyme was stimulated by Ca^{2+} and preferred pectin indicated that the pectolytic enzyme was classified as an endo-PMTE (Table 1). Whether other pectolytic enzymes, besides endo-

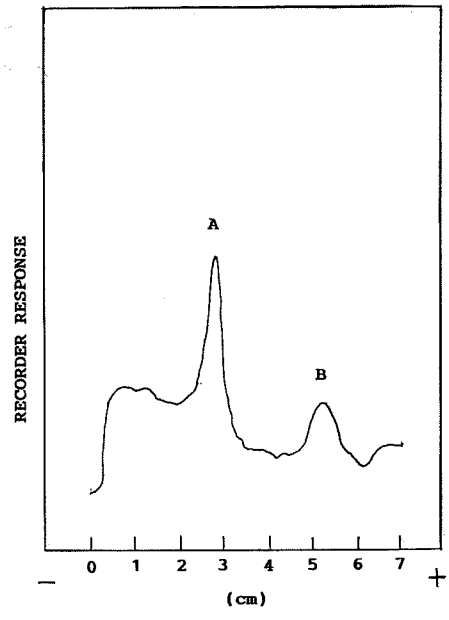

Fig. 8. Separation of endo-pectin methyl *trans*-eliminase a and b of *Ganoderma lucidum* by disc-gel electrophoresis (the purified endo-PMTE from Fig. 7). The electrophoretic gel was scanned by Kratos spectrodensitometer at 580 nm.

Table 2. *Purification of* ***Ganoderma lucidum*** *B endo-pectin methyl* ***trans****-eliminase*

Purification step	Volume (ml)	Protein content (mg/ml)	Total activity (units)	Specific activity (unit/mg protein)	Recovery (%)
Crude enzyme	100	2.61	506	1.94	100
Ammonium sulfate fractionation (60-80%)	15	5.62	267	3.17	52.7
Sephadex G-100 chromatography	30	3.00	580	6.46	24.2
DEAE cellulose chromatography	15	0.66	181	18.33	11.9
Isoelectric focusing					
endo PMTE a	5	0.40	39	19.30	7.6
endo PMTE b	5	0.47	44	19.10	8.9

Table 3. *Effects of crude and ammonium sulfate fraction of* ***Ganoderma lucidum*** *B pectolytic enzymes on potato tissue*

Treatment (h)	pH														
	Crude						60-80% $(NH_4)_2SO_4$ fraction						Autoclaved		
	4.5	7.0	8.0	4.5 (with 1mM $CaCl_2$)	7.0	8.0	4.5	7.0	8.0	4.5 (with 1mM $CaCl_2$)	7.0	8.0	4.5	7.0	8.0
3	0[1]	0	0	0	0	0	2	1	2	2	1	2	0	0	0
6	1	0	1	1	0	1	4	2	3	4	2	4	0	0	0
9	2	1	2	2	0	2	5	3	4	5	3	5	0	0	0

[1] 0---> no maceration; 5---> complete maceration.

PG and endo-PMTE which exhibit its enzyme activities at pH 6.5, also presented in our crude enzyme preparations remained to be elucidated.

In the current study, we found endo-PMTE activities of *G. lucidum* have two pH optima of 4.5 and 8.0. This is an ambiguous results because the optimum pH of the enzyme activity was normally found at alkaline range (Ochuchi and Tominaga, 1974; Cooper *et al.*, 1978; Hislop *et al.*, 1979; Tsuyumu and Chatterjee, 1984). However, Edstrom and Phaff (1964) reported that *Aspergillus fonsecaeus* pectin *trans*-eliminase has a optimum pH at 5.2 and Ca^{2+} inhibited the enzyme activity.

The endo-PMTE a and endo-PMTE b, obtained from polypropylene with mycelia of *G. lucidum* B, were purified by 60-80% ammonium sulfate fractionation, Sephadex G-100 gel filtration, DEAE cellulose ion exchange column chromatography and isoelectric focusing, showing pI at 8.2 and 5.5. Disc gel electrophoresis confirms two peaks corresponding to endo-PMTE a and endo-PMTE b as isoenzymes. These pectolytic enzymes were purified from *G. lucidum* B and the specific activity of endo-PMTE was only increased by 10 folds. The reasons why we failed to get a substantially increase in the enzyme specific activity throughout our purification procedure are speculated. We are suspicious that proteinases may degrade the endo-PMTE in our crude enzyme preparations and also ammonium sulfate may not be an adequate for precipitating endo-PMTE. This is substantially by the fact that, in 60-80% ammonium sulfate fraction of the crude endo-PMTE extract, only 52.7% of the enzyme activity was recovered. Furthermore, purification the

ammonium sulfate fraction through a Sephadex G-100 column, DEAE cellulose column and finally isoelectric focusing, apparently did not result in significant increase in the specific activities (shown in Table 2). It is due to the fact that proteinases do exist in our partially purified endo-PMTE from gel filtration column (data not shown). Thus the recovery of the enzyme decreased from 24.2 to 11.9% as shown in Table 2. Further investigations are needed before we can get a better system for the enzyme purification.

Research on pectolytic enzymes from *G. lucidum* was still meager. Two pectolytic enzymes endo-PG and endo-PMTE were isolated from the crude enzyme extracts of *G. lucidum* B from mycelia polypropylene bags. Kumari and Sirsi (1971) also reported that an endo-PG secreted by the fungus. This is the first report indicated that *G. lucidum* produced two isoenzymes of endo-pectin methyl *trans*-eliminase from the extracts of mycelia polypropylene bags. The enzyme, which is able to macerate potato discs *in vivo*, strongly suggests it plays an important role in relation to plant pathogensis.

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靈芝之研究
(三) 果膠質分解酶

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菌種 *Ganoderma lucidum* B 接種在液體培養液和太空包中，經測試有果膠質分解酶產生。從太空包培養基萃取液中，可純化出2種果膠質分解酶，即 endo-polygalacturonase (endo-PG) 及 endo-pectin methyl *trans*-eliminase (endo-PMTE)。其中 endo-PMTE 有2個最適 pH 值是 4.5 和 8.0，Ca^{2+} 可促進其作用，且僅偏好 pectin 爲其基質，溫度上升達 50°C 以上時，此酶漸失去其活性。

G. lucidum B 的太空包培養菌絲，經過 60-80% 硫酸胺分劃法，Sephadex G-100, DEAE cellulose 離子交換層析及等電集中法，可以純化出 endo-PMTE a 和 endo-PMTE b，其 PI 爲 8.1 和 5.5，並經不連續膠體電泳法 (Disc gel electrolysis)，證明有2高峯，故確認 endo-PMTE a 和 b 爲同功酵素。經純化後，endo-PMTE 的比活性爲原來的10倍。

由 60-80% 硫酸胺分劃法純化所得之果膠質分解酶，可以軟化馬鈴薯片，而且活性較粗果膠質分解酶還高。在 pH 4.5 時，這些酶對馬鈴薯片的分解能力，較之在 pH 8.0 或 7.0 時佳。pH 8.0 時 Ca^{2+} 可促進果膠質分解酶的活性，而且可以加速軟化作用。這是有關於 *G. lucidum* 產生 endo-PMTE 的首次報告。