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Properties of Amylase produced from Higher Fungi *Ganoderma lucidum*

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藥用担子菌類 靈芝가 生産하는 Amylase의 酵素学的 性質

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Extracellular amylase from the filtrate of the submerged culture of *Ganoderma lucidum* was partially purified by ammonium sulfate precipitation and its properties were studied.

The optimum pH and temperature of the enzyme activity were 5.5 and 50°C, respectively. This enzyme was most stable at pH 5.0 and stable up to 30°C, but it lost completely the activity when it was treated at 60°C for 10 min. The enzyme was activated by the addition of Mn^{++}, C^{++} and Cu^{++}, but inhibited by Hg^{++}, Ag^{+}. And various enzyme inhibitors and chemical reagents did not affect the enzyme activity.

The enzyme hydrolyzed the boiled amylaceous polysaccharides, but it hydrolyzed raw starches very slowly. The activation energy of the enzyme for soluble starch was calculated and found to be 7.06 Kcal per mole. The *Km* values of the enzyme for soluble starch, amylose, amylopectin and glycogen were 0.16, 0.37, 0.19, and 0.16mg/ml, respectively.

Maltose was found to inhibit the enzyme activity and kinetic analysis revealed a competitive type of inhibition.

It is generally recognized that microorganisms have various kinds of enzymes in their cells. Each enzyme has a special mode of action with its own substrate specificity. The glucohydrolase system of microorganisms, particularly thermostable or thermophilic amylase, has been intensively studied by many works and many kinds of these enzymes have been found to function in different manners[1-4]. But there were few studies on enzymes from *Basidiomycetes*, except that ATP-sensitive and ATP-insensitive ribonuclease[4], alkaline phosphodiesterase[5], proteolytic and milk clotting enzyme[6], macerating enzyme[7], α -D-glucosidase and glucoamylase[8], amylase[9] and other nucleases[10,11] were reported.

Ganoderma lucidum is a *Basidiomycetes* which can produce a fruit body in its life cycle. It produces various chemical compounds, free- monosaccharide, sugar-alcohol, oligosaccharide, amino acid, steroid, triterpenoid, cumalin glycoside, protein, polysaccharide[12-16] and antitumor polysaccharide having α, β-(1→3)-linked backbone and branch β-(1→6)-linkages[17-19]

Kawai[20] reported the studies on the productivity and distribution of amylolytic, cellulolytic and xylolytic enzymes produced by submerged culture of *Basidiomycetes*. By the result, amylolytic, trehalase and C_2 enzyme activity produced by *Ganoderma* were very low, but Cx enzyme and xylanase showed very high activities.

We found that a strain belong to *Ganoderm lucidum* accumulates a large amount of amylolytic enzyme in the medium under certain condition. This paper describes the properties and some kinetics of the extracellular amylolytic enzyme obtained from the submerged culture of *Ganoderma lucidum*.

Materials and Methods

Microorganism and culture conditions

A strain, *Ganoderma lucidum* used in this experiment was provided by Chung-ang Agriculture Enterprise Co., and the strain was maintained on potato-dextrose agar medium.

The medium for amylase production was potato-dextrose broth, and the initial pH of the medium was adjusted to 5.5. The cultivation was carried out in a 500ml aerobic Erlenmeyer flask containg 100ml of the medium on shaking incubator (200rpm) at 30°C for 14 days.

Preparation of enzyme solution

Culture solution was filtered by Toyo No.2 filter paper and the filtrate was saturated with ammonium sulfate, subsequently stored at 4°C for 12hrs. The precipitate was collected by centrifugation (5000rpm, 30mim) and disolved in McIlvaine buffer (pH 5.5), then dialyzed against the same buffer at 4°C for 1 day. After filteration, it was used as crude enzyme solution.

Assay of amylase activity

Amylase activity was measured in a reaction mixture contained 0.5ml of McIlvaine buffer solution (pH 5.5), 0.4ml of 1.0% soluble starch disolved in same buffer solution and 0.1ml of suitably diluted enzyme solution. The reaction mixture was incubated at 40°C for 10min and reducing sugars released during incubation were determined by DNS method[21]. One unit of amylolytic activity is defined as 1mM of glucose released/ml/min using soluble starch as substrate.

Kinetic properties of the enzyme

Reaction mixtures of various concentrations of soluble starch, amylose, amylopectin and glycogen with enzyme solution were reacted at 50°C for 10min. After reaction, the amounts of glucose released from the substrates were determined as described in the enzyme assay. *Km* values for substrates were obtained from the method described by Lineweaver and Burk[22].

Results and Discussion

Effect of pH on enzyme activity

The effect of pH dependence and stability of amylase from *Ganoderma lucidum* were examined at 40°C and the results are shown in Fig. 1. The optimum pH of the enzyme activity was found to be about pH 5.5 and the enzyme was most stable at pH 5.0. The optimum pH on the enzyme activity is similar to the results reported for *Thermoactinomyces vulgaris*[23], *Schizophyllum commune*[9] and *Lentinus edodes (Berk.) Sing*[8]. But it showed slightly higher value than that of glucoamylase from *Lentinus edodes (Berk.) Sing*[8]. The pH range for stability is more narrow than those of the amylase from *Bacillus sp.*[1,2] and glucoamylase and α-glucosidase from *Lentinus edodes (Berk.) Sing.*[8].

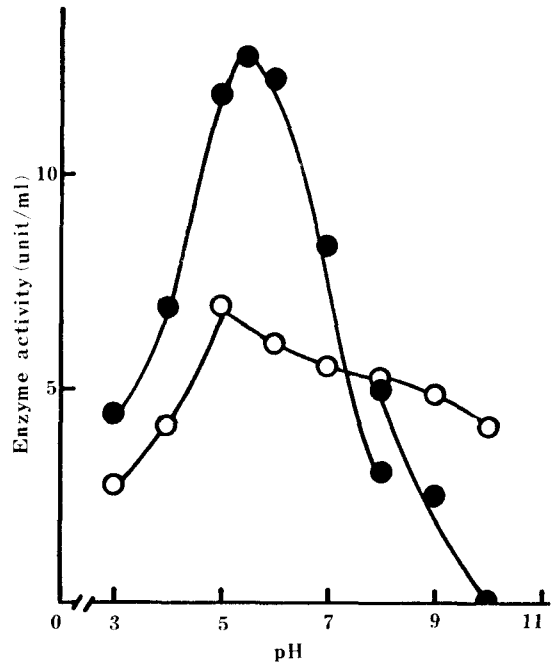

Fig. 1. Effect of pH on enzyme activity.

Enzyme activity (●) was determined at various pH value; McIlvaine buffer (pH 3 − 8), Clark and Lubs buffer (pH 8 − 10). Remaining activity (○) was measured after treatment at 40℃ for 1hr at various pH values.

Effect of temperature on enzyme activity

The enzyme activity was measured in range of temperature between 20°C and 70°C at pH 5.5. The optimum temperature was about 50°C and stable up to 30°C. This enzyme was heat-unstable enzyme which its activity was completely lost by heat treatment at 60°C for 10min (Fig. 2). The optimum temperature and thermal stability of the enzyme is lower than values and stability of the amylases from *Bacillus stearothermophilus*[3], *Thermophilic bacterium*[24], *Thermomonospora vulgaris*[25] and *Thermoactinomyces vulgaris*[23].

By the means of Arrhenius' equation, the energy of activation of dextrinogenic reaction was calculated and found to be 7.06 Kcal/mole and the temperature coefficient (Q_{10}) was found to be 1.44 between 20°C and 30°C, 1.36 between

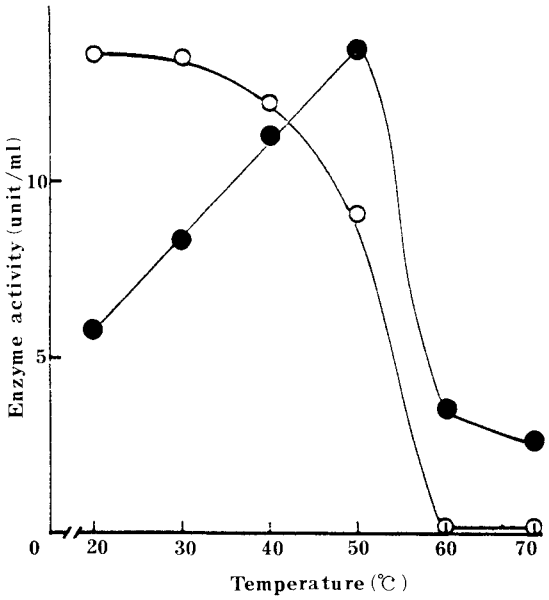

Fig. 2. Effect of temperature on enzyme activity.

Enzyme activity (●) was determined at various temperature (pH 5.5) and remaining activity (○) was measured after treatment at various temperature (pH 5.5) for 10min.

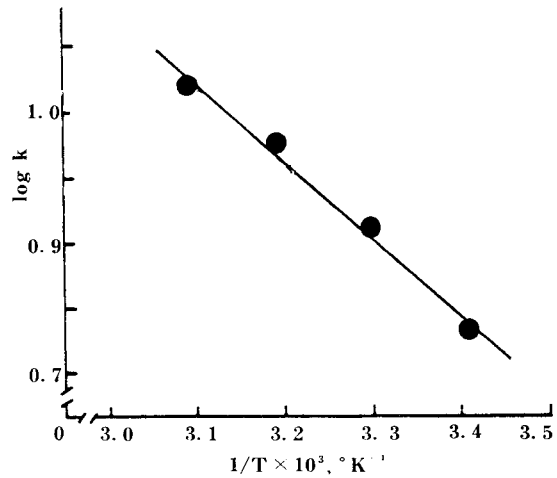

Fig. 3. Arrhenius plot for the amylolytic reaction of the enzyme.

30°C and 40°C, and 1.22 between 40°C and 50°C (Fig. 3). This result indicates that the enzyme denaturation occurred quickly at higher temperature.

Effects of metal ions and chemical reagents on the enzyme activity

In order to investigate the effects of various metal ions and chemical reagents on the amylase activity, the enzyme solution and substrate was incubated with 1mM metal ions and chemical reagents at pH 5.5, 50°C for 10min. The residual activity of the enzyme was determined as described in Materials and Methods and the results are shown in Table 1,2. The enzyme was activated by the addition of 1mM

Table 1. Effect of metal ion on enzyme activity

Metal	Activity (unit/ml)	Relative activity (%)
None	12.22	100
$BaCl_2 \cdot 2H_2O$	11.67	94
$AlCl_3 \cdot 6H_2O$	11.94	97
$CoCl_2 \cdot 6H_2O$	13.89	116
$FeSO_4 \cdot 7H_2O$	12.78	106
$MgSO_4 \cdot 7H_2O$	12.33	102
$ZnSO_4 \cdot 7H_2O$	12.77	105
$MnCl_2 \cdot 4H_2O$	16.94	146
$Pb(NO_3)_2$	13.33	111
$CaCl_2 \cdot 2H_2O$	15.00	127
$CuSO_4 \cdot 5H_2O$	15.28	131
$HgCl_2$	6.11	39
$AgNO_3$	8.06	59
$Li_2SO_4 \cdot H_2O$	12.32	101

Metal ion was added with the concentration of 1mM in the reaction mixture.

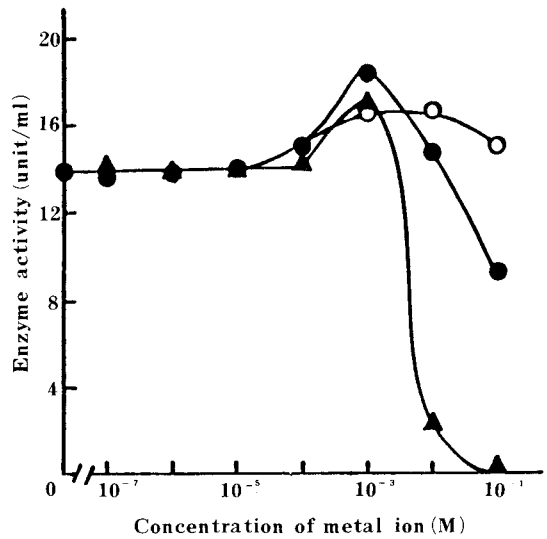

Fig. 4. Effect of metal ion concentration on enzyme activity.

●; Mn^{++}, ○; Ca^{++}, ▲; Cu^{++}

Table 2. Effect of chemical reagent on enzyme activity.

Chemical reagent	Activity (unit/ml)	Relative activity (%)
None	12.50	100
Thiourea	11.67	92
p-CMB	10.83	85
Urea	12.22	96
Sodium Arsenate	12.24	98
ε-Amino-*n*-caproic acid	12.50	99
SDS	12.40	99
MIA	12.78	103
Sodium Azide	12.60	100
Sodium Fluoride	12.76	103
o-Phenanthroline	12.60	100
2,4-DNP	11.67	95
L-Cystine	13.06	104
EDTA-2Na	11.22	87

Chemical reagent was added with the concentration of 1mM in the reaction mixture.

Mn^{++}, Ca^{++} and Cu^{++}, on the other hand inhibited by Hg^{++}, Ag^{+}. Ca^{++} is concerned with enzyme stabilization or activation[26,27] and Cu^{++} inactivated the enzyme activity[24] but this enzyme was significantly stimulated by the addition of Cu^{++} in the reaction mixture (Fig. 4) and the enzyme activities at various concentrations of metal ions were shown as Fig. 4. All chemical reagents did not affect enzyme activity (Table 2).

From this results, we suggest that this enzyme is not a metalloenzyme but a metal activated enzyme which has not any metal ion as prosthetic group in enzyme molecule.

Substrate specificity and *Km* value

The enzyme activity was examined with various substrate. The results are summarized in Table 3. As shown in Table, this enzyme could hydrolyzed soluble starch, amylose, amylopectin, and glycogen. Although the enzyme hydrolyzed raw starches, the rate of hydrolysis was much lower than those for boiled amylaceous polysaccharides.

The effect of substrate concentrations on the reaction velocities were examined. The *Km* value of the enzyme for soluble starch, amylose, amylopectin and glycogen were calculated from the initial rate based on a Lineweaver-Burk reciprocal plot[22] to be 0.16, 0.37, 0.19 and 0.16mg/ml, respectively (Fig. 5). These value were much lower than the values obtained for α-amylase from other microoganisms[3,25,28].

Table 3. Substrate specificity of enzyme activity

Substrate	Activity (unit/ml)	Relative activity (%)
Soluble starch	13.05	100
Amylose	13.61	108
Amylopectin	8.89	63
Glycogen	11.11	81
Corn starch	13.05	100
Potato starch	13.10	101
Corn starch*	3.89	16
Potato starch*	2.50	3

*Starches were not treated with boiling water.

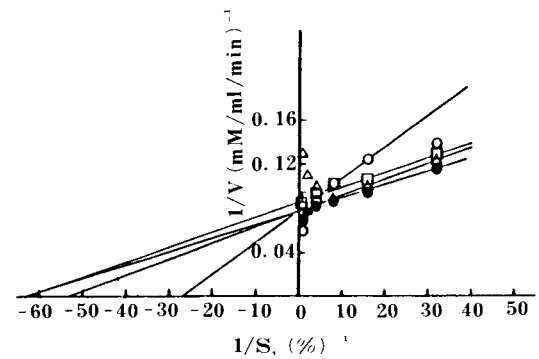

Fig. 5. Lineweave-Burk plot of starch hydrolysis by the enzyme.

●; soluble starch, ○; amylose, △; amylopectin, □; glycogen

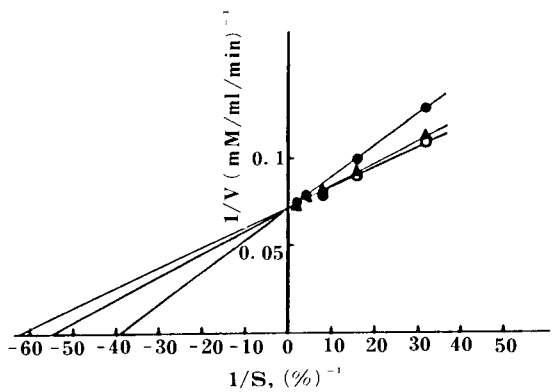

Fig. 6. Double reciprocal plots for hydrolysis of soluble starch by the enzyme in the presence or absence of maltose.

○; in the absence of maltose, ▲; and ●; in the presence of 1mM and 2.5mM maltose in the reaction mixture respectively.

Table 4. Effect of various sugars on enzyme activity

Sugars	Activity (unit/ml)	Relative activity (%)
None	7.94	100
Sucrose	7.39	89
D-Fructose	6.67	78
Maltose	5.83	65
D-Xylose	7.22	86
D-Glucosamine·HCl	7.61	95
Lactose	7.44	90
D-Glucose	7.44	90
α-L-Rhamnose	7.78	98
L-Arabinose	7.63	96
i-Inositol	7.78	98
Raffinose	7.94	100
D(+) Cellobiose	7.50	91
D(+) Galactose	7.78	98
D( ) Ribose	7.78	98
D-Galacturonic acid	7.96	100
α-D(+) Melibiose	7.78	98
β-Gentiobiose	7.61	95
D(+) Melezitose	7.80	99
Maltotriose	6.94	83
Maltotetraose	7.50	91

Soluble starch and sugar were added with the concentration of 0.03125% and 2mM in the reaction mixture.

Effect of various sugars on the enzyme activity

In order to study the effects of metabolites of the substrate or other sugars on the enzyme activity, each sugar was added with the concentration of 2mM in reaction mixture and reacted at pH 5.5, 50°C for 10min. As shown in Table 4, the enzyme action was depressed by the addition of maltose, fructose, xylose, sucrose, maltotriose, and maltotetraose. In Fig. 6 are shown Lineweaver-Burk plots in the presence and absence of maltose. The *Km* values in the presence or absence of maltose were different, indicating that inhibitory action of maltose on the enzyme was competitive.

요 약

Ganoderma lucidum (靈芝)의 액체 배양물로부터 전분을 강력하게 분해하는 amylase를 조정제하여 이 효소의 기본적인 성질을 조사하였다.

이 효소의 최적작용 pH와 온도는 각각 5.5, 50℃였고 pH 및 열처리에 상당히 불안정한 효소였으며 activation energy는 7.06Kcal/mole이었다. Mn^{++}, Ca^{++} 및 Cu^{++}에 의해서 효소활성이 증가되었으나 Hg^{++}, Ag^{+}에 의해서 효소활성이 저해되었으며 여러가지 chemical reagents에 의해서는 영향을 받지 않았다.

Soluble starch, amylose, amylopectin 및 glycogen에 대한 *Km* 치는 0.16, 0.37, 0.19 및 0.16mg/ml였으며 각종기질에 대한 분해능력을 조사한 결과 열처리를 받은 전분류는 분해할 수 있었으나 생전분은 그 분해속도가 느렸다. Maltose에 의해서 효소활성이 저해되었으며 maltose의 저해양상은 competitive inhibition을 나타내었다.

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