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武庫川女子大紀要（自然科学）

Cultural characteristics of *Laetiporus sulphureus*, producing an anti-thrombin substance

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The clotting time for thrombin of the culture broth from *Laetiporus sulphureus* was more than 44 times that of the control(2% malt extract medium), whereas the culture broths of all other strains exhibited lower anti-coagulative activity. In experiments on the utilization of carbon sources, the mycelial growth of *L. sulphureus* was rapid with glucose as the sole carbon source. Casamino acid was the best source for the strain. Thiamine was required for the mycelial growth of *L. sulphureus*. The optimum temperature for efficient mycelial growth was recorded at 30℃. The initial pH 4 to 5 was the most favorable for *L. sulphureus* for growth.

Introduction

Thrombosis is one of the major causes of death in Japan and the western world. Three thrombolytic agents clinically used at present are streptokinase, urokinase and tissue-type plasminogen activator(t-PA). However, there still exist major problems with their use in terms of antigenicity, non-specific breakdown of other blood factors and localization of the fibrin clot because of high enzymatic activity[1),2)]. On the other hand, anticoagulants of natural origin, such as heparin and antithrombin Ⅲ, have been found in constituents of the human body[3)]. Antithrombin Ⅲ is an effective antithrombotic agent, but its activity depends on the coexistence of heparin, and it is a macromolecule substance. Okamoto *et al.* reported a synthetic thrombin inhibitor(argatroban), 4-methyl-1-(N^2-3-methyl-1, 2, 3, 4-tetrahydro-8-quinolinylsufonyl-L-arginyl)-2-piperidinecarboxylic acid[4),5)], which is an effective drug for cerebral thrombosis in its acute stage[6)]. But this compound is unlikely to be satisfactory as a thrombolytic agent in the chronic stage. Therefore, the screening of inhibitory agents for thrombin which correspond to the formation of fibrin, but not that of plasminogen activators, and fibrinolytic enzymes is now desirable. We screened for a basidiomycetes anti-thrombin substance. In this study, basidiomycetes with an anti-thrombin substance was discovered from a culture broth of basidiomycetes, and the cultural characteristics of *L. sulphureus* which also produces an anti-thrombin substance was also described.

Materials and Methods

1. Organisms

Forty-one strains of wild basidiomycetes isolates were isolated from Kitakomatsu, Shiga and Ashibidani, Shiga prefecture, Japan. Cultures of wild basidiomycetes isolates were obtained by aseptic inoculation of the tissue from developing fruit bodies onto media containing 2% malt

extract.

2. Medium for basidiomycetes

The basidiomycetes were cultured in 300-mL Erlenmyer flasks with 100 mL of 2% malt extract medium at 25℃ for 2 weeks on a rotary shaker(80 rpm).

3. Culture experiments for nutrition and physical conditions

W0008(*Laetiporus sulphureus*)was cultured separately in test tubes with 6 mL of the previously autoclaved basal medium containing 20g D-glucose, 2g peptone, 0.5g KH_2PO_4, 0.1g $CaCl_2$ and 100 μg thiamine hydrochloride in 1 liter of distilled water. The inoculum consisted of 0.1 mL from a fully grown culture which was previously blended and macerated. Incubation of cultures was done at 25℃ under stationary conditions for 10 days. The mycelial weight was obtained by filtering through a fine sieve, and washing and drying in an oven at 100℃ for 15 hours before weighing. For the tissue culture, a 2% malt extract agar plate was used.

4. Coagulability test

Coagulability was measured using the method described by Kinoshita and Horie[8], in the following way:(1)the activated partial thromboplastin time(APTT), the time elapsing until plasma clots in the presence of crude phospholipids(and therefore measurement of the intrinsic clotting pathway);(2)the prothrombin time(PT), the time elapsing until plasma clots in the presence of tissue thromboplastin(and therefore measurement of the extrinsic clotting pathway); and(3)the thrombin time(TT), the time elapsing until the fibrin formation of thrombin.

After growth, the culture broth was centrifugated to remove the mycelia. The supernatant obtained was tested for thrombin activity. Bovine α-thrombin was purchased from Mochida Pharmaceutical Co., Ltd. Whole blood was from the vena of Jol-ICR mice.

The citrated plasma was used to measure clotting time. The clotting time of thrombin in a reaction mixture(37℃)containing 50 μL of the culture broth, 50 μL of 12.5 NIH unit/mL thrombin and 200 μL of 0.33% bovin fibrinogen was measured with a KC1A coagulometer(Henrich Amelung)[9].

Results and Discussion

1. Effect of various media on TT

One culture broth from W0008, which was identified as *Laetiporus sulphureus* by Syoithi Yoshimi who is a mushroom researcher, showed anti-coagulative activities on TT. A photograph of *L. sulphureus* is shown in Fig. 1.

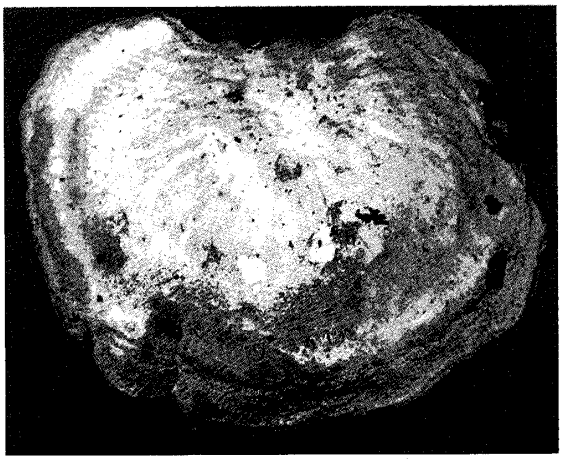

Fig. 1. Photograph of *Laetiporus sulphureus* (W0008).

The clotting time for TT of the culture broth from *L. sulphureus*(more than 200 seconds)was more than 44 times that of the control(4.5 seconds [2% malt extract medium]), whereas the culture broths of all other strains exhibited lower anti-coagulative activity(data was not shown).

The APTT and PT of the culture broth from *L. sulphureus* were 34.1 seconds and 24.0 seconds, respectively. On the other hand, the APTT and PT of the control were 19.3 seconds and 15.3 seconds, respectively.

2. Nutritional requirements of the mycelium

In the experiments on the utilization of carbon sources, the mycelial growth of *L. sulphureus* was rapid with glucose as a sole carbon source, followed by fructose, galactose, mannitol, mannose, maltose, sucrose and starch. As nutrients, monosaccharides or disaccharides

served better than polysaccharides as carbon sources. Neither xylose, arabinose, ethanol, citrate or malate served as good carbon sources for *L. sulphureus*(Table 1.).

Table 1. Effect of carbon sources on mycelial growth.

Carbon source (2%)	Mycelium (mg/6mL)
Mannose	2.1
Starch	1.9
Glucose	2.3
Maltose	2.1
Sucrose	2.1
Fructose	2.1
Mannitol	2.0
Galactose	2.0
Xylose	trace
Arabinose	0.1
Ethanol	trace
Citrate	0.5
Malate	trace
None	0

The basal media in which glucose was replaced with carbon sources are as indicated. Incubation was done at 25℃ for 10 days.

To investigate good sources of nitrogen for *L. sulphureus*, peptone in the basal medium was replaced by its nitrogen equivalent from other sources. Casamino acid was the best source for the strain. Peptone also promoted good growth, followed by alanine, serine and asparagine. The organic nitrogen sources may equally serve carbon and nitrogen nutrition. Ammonium chloride, like the other inorganic acid ammonium salts, also promoted growth. On the other hand, potassium nitrate and calcium nitrate were not suitable as nitrogen sources for *L. sulphureus*(Table 2.).

Table 2. Effect of nitrogen sources on the mycelial growth.

Nitrogen source (0. 2gN/g)	Mycelium (mg/6mL)
Peptone	2.3
Casamino acid	3.5
Asparagine	1.5
Serine	1.8
Alanine	1.9
Phenylalanine	trace
Methionine	trace
Proline	trace
Ammonium sulfate	0.4
Ammonium chloride	0.8
Ammonium nitrate	0.2
Potassium nitrate	trace
Calcium nitrate	trace
None	trace

The basal media in which peptone was replaced with other sources of nitrogen are as indicated. Incubation was done at 25℃ for 10 days.

Table 3. shows the results for the vitamin requirements for the mycelial growth of *L. sulphureus*. Thiamine at 100 μg/L was required for the mycelial growth of *L. sulphureus*. The addition of other vitamins such as riboflavin and vitamin B_{12} in the presence of thiamine had an influence on growth. There was also a response to biotin, niacin, pyridoxine, riboflavin and vitamin B_{12} without the addition of 100 μg/L thiamine.

Table 3. Effect of thiamine concentration and other vitamins on mycelial growth.

Thiamine (μg/L)	Vitamin (μg/L)		Mycelium (mg/6mL)
1,000	–		2.4
500	–		2.3
100	–		2.3
10	–		2.0
1	–		1.9
0.1	–		1.7
0	–		0.9
100	Biotin	100	2.3
100	Niacin	100	2.3
100	Pyridoxine	100	2.3
100	Riboflavin	100	2.6
100	Vitamin B_{12}	100	2.5
–	Biotin	100	1.1
–	Niacin	100	2.0
–	Pyridoxine	100	1.6
–	Riboflavin	100	1.4
–	Vitamin B_{12}	100	1.3

Incubation was done at 25℃ for 10 days.

3. Environmental conditions for growth

Table 4. shows the effect of temperature on the mycelial growth of *L. sulphureus*. Mycelial growth was noted even at 35℃. However, the growth was quite abnormal at this temperature.

The optimum temperature for efficient mycelial growth was recorded at 30℃.

No growth occurred at an initial pH of 12.0. Mycelial growth occurred in a pH ranging from 3.0 to 11.0. The initial pH 4 to 5 was most favorable for *L. sulphureus* (Table 5.).

Table 4. Effect of temperature on mycelial growth.

Temperature (℃)	Average diameter of colonies (cm)
15	1.6
20	3.8
25	6.1
30	8.5
35	4.9

After 10 days, the extent of growth was measured on a 2% malt extract agar plate.

Table 5. Effect of pH on mycelial growth.

Initial pH	Average diameter of colonies (cm)
3.0	7.0
4.0	8.2
5.0	7.5
6.0	7.3
7.0	7.1
8.0	7.1
9.0	7.1
10.0	6.8
11.0	6.4
12.0	0

After 8 days at 25℃, the extent of growth was measured on a 2% malt extract agar plate.

Acknowledgments

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