The Production and Stability of Intracellular Myrosinase from *Aspergillus niger*[†]

Masaru OHTSURU, Isao TSURUO and Tadao HATA

Research Institute for Food Science, Kyoto University, Kyoto

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After Screening 100 micro-organisms to detect intracellular myrosinase, only *Aspergillus niger* produced myrosinase.

Enzyme production was induced by the addition of ten percent of a mustard extract* to the culture medium. The enzyme was produced in considerable amounts on the first and second day of cultivation. L-Ascorbic acid was an excellent carbon source.

The enzyme was unstable but was stabilized by coexistence with 2-mercaptoethanol (10^{-2} M) and ascorbic acid (10^{-3} M).

In previous papers,^{1~3)} the production, purification and various properties of myrosinase produced by *Aspergillus sydowi* were described. This is extracellular enzyme which is induced by a mustard extract during about 2 weeks of cultivation. It is very difficult to obtain large amounts of the enzyme, because of the long period necessary to cultivate the organism.

Reese *et al.*⁴⁾ reported that the myrosinase (sinigrinase) produced by *Aspergillus sydowi* is an extracellular enzyme; sinigrinase was always found slightly in mycelial extract when it was present in the culture filtrate, and never found in mycelial extract when it was absent in the culture filtrate. The nature of intracellular myrosinase is still undetermined.

In the present experiment, we surveyed organisms that produce myrosinase in their mycelia in hopes of obtaining a large amount of enzyme while shortening the culture periods. Enzyme activity was found in the mycelia of *Aspergillus niger*.

Culture conditions for the fungus and enzyme stability are described. Some chara-

cteristics of the myrosinase from *Asp. niger* are also discussed in comparison with those of the enzyme from *Asp. sydowi*.

MATERIALS AND METHODS

Organisms. Various stems of fungi and bacteria were kindly supplied by the Faculty of Agriculture of Kyoto University.

Culture conditions. Culture conditions for screening were as follows. Organisms (80 bacteria and 20 fungi) were inoculated in a medium containing 20% mustard extract, 0.1% glucose, 0.1% yeast extract, 0.1% potassium dihydrogen phosphate, 0.1% ammonium sulfate and 0.05% magnesium sulfate (as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), pH 6.5.

Fungi were cultivated for 2 weeks and bacteria for 2 days at 29°C under shaking.

Aspergillus niger AKU 3302 was pre-cultured at 29°C in 100 ml of medium containing 5% malt extract, 2% sucrose, 0.2% potassium dihydrogen phosphate, 0.1% ammonium sulfate, 0.03% sodium nitrate, and 0.03% magnesium sulfate (as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), pH 6.5. After incubation for 2 days, mycelia were harvested and washed twice with water. Wet mycelia were inoculated in 100 ml of synthetic medium consisting of 0.1% yeast extract, 0.2% potassium dihydrogen phosphate, 0.1% ammonium sulfate, 0.03% sodium nitrate, 0.03% magnesium sulfate, 0.1% glucose and 20 ml of mustard extract, unless otherwise stated. Cultivation was carried out in 500 ml flasks containing 100 ml of the medium for 2 days at 29°C under shaking on a reciprocal shaker. Approximately 5~6 g of mycelia (wet weight) were obtained per 100 ml of the pre-culturing medium. In all cultivating experiments,

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* Extract of mustard seed (1.0 kg) with 80% methanol (5.0 liters) was concentrated to 700 ml.

about 5 g of mycelia (wet weight) were inoculated in 100 ml of induction medium.

Substrate. Sinigrin was prepared from yellow mustard seed as described previously,^{5,6)} and used as the substrate of myrosinase.

Enzyme preparation. After cultivation, contents of the flasks were filtered and washed twice with water. Approximately 5~6 g of wet mycelia were ground together with sea sand and 0.2 M phosphate buffer (pH 7.0, 15 ml), and then it was centrifuged. The supernatant was used in experiments as the crude preparation.

Enzyme assay. Enzymatic activity was determined as β -thioglucosidase activity. The enzyme reaction was carried out in a system containing 2.5 μ moles of substrate and 0.1 μ mole of phosphate buffer, pH 7.0, in a total volume of 1 ml. The reaction was carried out at 37°C and at pH 7.0, unless otherwise stated. Thioglucosidase activity was measured by the liberation of glucose. The reaction was conducted under conditions regarded as a zero order reaction.⁵⁾

Total activity. The wet mycelia were ground together with sea sand and 0.2 M phosphate buffer (pH 7.0), and then it was centrifuged. The supernatant was filled up to 50 ml with the same buffer. Total activity was expressed as μ moles of glucose per minute per 50 ml unless otherwise stated.

RESULTS

Survey of organisms

Results of the screening test are summarized in Table I. Enzyme activity was determined by using the supernatant of ground mycelia with sea sand for fungi and the soluble part after sonication for bacteria. Strong activity was found in the mycelia of *Asp. niger* AKU 3302 but only slight activity was detected in *Asp. sydowi* IFO 4284. The same test was carried out with some others strains of *Asp. niger* group (Table II); all the strains showed

TABLE I GROWTH OF BACTERIA AND FUNGI ON A MUSTARD EXTRACT MEDIUM

Organisms were inoculated in a medium containing 20% mustard extract, 0.1% glucose, 0.1% yeast extract, 0.1% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$ and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5.

Fungi were cultivated for 2 weeks and bacteria for 2 days at 29°C under shaking.

	Growth	Myrosinase activity
<i>Pseudomonas aeruginosa</i> (IFO 3080)	+++	—
<i>Sarcina lutea</i> (IAM 1099)	+++	—
<i>Staphylococcus aureus</i> (IFO 3061)	—	—
<i>Flavobacterium fuscus</i> (AKU 0140)	—	—
<i>Micrococcus lysodeikticus</i> (IFO 3333)	+	—
<i>Alcalygenes faecalis</i> (IAM B-141-1)	—	—
<i>Proteus vulgaris</i> (IFO 3167)	—	—
<i>Serratia marcescens</i> (IFO 3046)	±	—
<i>Aerobacter aerogenes</i> (IFO 3320)	+++	—
<i>Escherichia coli</i> (AKU 0001)	—	—
<i>Bacterium cadaveris</i> (IFO 3731)	±	—
<i>Bacillus subtilis</i> (IFO 3026)	+++	—
<i>Streptococcus faecalis</i> (IFO 3181)	—	—
<i>Candida utilis</i> (IFO 0396)	+++	—
<i>Aspergillus</i>		
<i>oryzae</i> (AKU 3301)	++	—
<i>niger</i> (AKU 3302)	+++	++
<i>fumigatus</i> (IFO 4040)	++	—
<i>flavus</i> (IFO 5839)	++	—
<i>sydowi</i> (IFO 4284)	++	—
<i>Penicillium</i>		
<i>notatum</i> (IFO 4640)	+++	—
<i>urticae</i> (IFP 7011)	+++	—
<i>Mucor rouzianus</i> (IFO 5773)	+	—
<i>Phizopus oryzae</i> (AKU 0021)	+++	—

TABLE II. MYROSINASE PRODUCTION BY SOME OF THE *Aspergillus niger* GROUP

Fungi were pre-cultured at 29°C on 100 ml of medium containing 5% malt extract, 2% sucrose, 0.2% KH_2PO_4 , 0.03% NaNO_3 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5, respectively. After incubation 2 days, mycelia were harvested and washed twice with water. Wet mycelia were inoculated in 100 ml of synthetic medium.

Organism		Myrosinase activity
<i>Aspergillus niger</i>	IFO 4034	++
"	IFO 4068	++
"	IFO 4280	+
"	IFO 6341	+
"	IFO 5374	++
"	AKU 3302	++

FIG. 1. Enzyme Production in Mycelia of *Aspergillus niger*.

The fungus was pre-cultured at 29°C on 500 ml of medium containing 5% malt extract, 2% sucrose, 0.2% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.03% NaNO_3 and 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5. After incubation for 2 days, mycelia (about 25 g) were harvested and washed twice with water. About 5 g of the wet mycelia were inoculated in each 100 ml of synthetic medium. Total activities were measured by the liberation of glucose as described in the text.

considerable activity. *Asp. niger* AKU 3302 was used in the following experiments.

Determination of the culture conditions

Production of intracellular myrosinase. The relationship between enzyme production and the culture period is shown in Fig. 1. The enzyme was produced in considerable amounts,

FIG. 2. Effect of Mustard Extract Concentration of Enzyme Production in Mycelia of *Aspergillus niger*.

The fungus was pre-cultured as described in Fig. 1. After incubation for 2 days, wet mycelia (weight about 25 g) were harvested and washed twice with water. About 5 g of the wet mycelia were inoculated in each 100 ml of synthetic medium, in which the concentration of mustard extract was changed. Total activities were measured by the liberation of glucose as described in the text.

mostly on the first and second days.

Concentration of mustard extract. The relationship between enzyme production and the concentration of mustard extract in the medium is shown in Fig. 2. The best enzyme production was observed with 10% mustard extract; production gradually decreased at the higher concentrations.

Asp. niger was pre-cultured and its mycelia were inoculated in the synthetic medium. The greatest amount of enzyme was produced in a medium containing 0.1% glucose, 0.1% C.S.L., 0.03% cobalt chloride (as $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) and 10% mustard extract. Corn steep liquor was a better nitrogen source and cobalt chloride was also superior to magnesium sulfate. Consequently, these culture conditions were used for the following experiments.

No enzyme was produced without the addition of inducer (mustard extract or sinigrin) in the case of *Asp. sydowi*. The same was true for *Asp. niger*. As shown in Table III, no enzyme was produced without mustard extract.

TABLE III. EFFECTS OF SOME SUBSTITUTES FOR MUSTARD EXTRACT

Aspergillus niger was pre-cultured at 29°C on 1000 ml of medium containing 5% malt extract, 2% sucrose, 0.2% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.03% NaNO_3 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5. After incubation 2 days, wet mycelia (about 50 g) were harvested and washed twice with water. About 5 g of the wet mycelia was inoculated in each 100 ml of medium containing 0.1% glucose, 0.1% C.S.L., 0.03% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and substitutes for mustard extract, pH 6.5.

Materials	Conc.	Myrosinase activity
Mustard extract	10 %	++
Malt extract	0.1	—
Glucose	0.1	—
Sucrose	0.1	—
Ascorbic acid	0.1	—
Cellobiose	0.1	—
Amygdalin	0.1	—
Tyramine	0.05	—

TABLE IV. EFFECTS OF VARIOUS CARBON SOURCES

Aspergillus niger was pre-cultured at 29°C on 500 ml of medium containing 5% malt extract, 2% sucrose, 0.2% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.03% NaNO_3 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5. After incubation 2 days, mycelia (about 25 g) were harvested and washed twice with water. About 5 g of the wet mycelia were inoculated in each 100 ml of medium containing 10% mustard extract, 0.1% C.S.L., 0.03% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and various carbon sources, pH 6.5.

Source	Conc. (%)	Total activity (μ moles of glucose/min/50 ml)
Sucrose	0.1	2.0
Glucose	0.1	4.5
"	0.2	4.3
Ascorbic acid	0.1	8.8
Sorbitol	0.1	1.6
Cellobiose	0.1	1.3

Effects of various carbon sources. Effects of various carbon sources on enzyme production were tested (Table IV). Ascorbic acid was more effective than glucose. From these results, we composed a medium containing 0.1% ascorbic acid, 0.1% C.S.L., 0.03% cobalt chloride and 10% of mustard extract, pH 6.5, for the induction medium.

Stability of the enzyme. Intracellular myrosinase from *Asp. niger* was very unstable after extraction from the mycelia and was considerably inactivated during dialysis and precipitation process with ammonium sulfate. Effects of various reagents to stabilize the enzyme were examined. 2-Mercaptoethanol (10^{-2} M) containing ascorbic acid (10^{-3} M) was an effective stabilizer on the activity of the crude enzyme (Table V).

DISCUSSION

To obtain enzymes from microbes, we must take into account many conditions, of which the culture period is one of the most important factors. Generally, microbes must be cultivated for longer periods to obtain extracellular enzymes than to obtain intracellular enzymes. For myrosinase¹⁾ from *Asp. sydowi*, the fungus must be cultivated in an induction medium for at least 10 days under constant maintenance of a neutral pH range. We searched for myrosinase in various microbial mycelia in order to obtain it in large amounts. We also tried to shorten the culture period. *Aspergillus niger*, AKU 3302 after screening 100 organisms, was found to produce the enzyme.

TABLE V. EFFECT OF VARIOUS REAGENTS ON STABILITY

Time (hr)		Conc. (M)		Control		Glucose		Ascorbic acid		CoCl ₂		Ascorbic acid (10^{-3}) + 2-Mercaptoethanol (10^{-2})	
						10^{-3} 10^{-2}		10^{-3} 10^{-2}		10^{-3}			
Relative Activity (%)	0		100	100	100	100	100	100	100	100	100	100	
	24 (5°C)		72	80	91	97	45	57				98	
	48 (20°C)		33	34	51	65	24	12				80	(80) ^{a)}
												(52) ^{a)}	

^{a)} only 2-mercaptoethanol (10^{-2} M)

FIG. 3. Comparison of Enzyme Production by *Aspergillus niger* and *Aspergillus sydowi*.

Aspergillus niger and *Asp. sydowi* were pre-cultured at 29°C on 500 ml of medium containing 5% malt extract, 2% sucrose, 0.2% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.03% NaNO_3 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5, respectively. After incubation for 2 days, mycelia (about 25 g) were harvested and washed twice with water. About 5 g of the wet mycelia of *Asp. niger* were inoculated in each 100 ml of medium containing 10% mustard extract, 0.1% ascorbic acid, 0.1% C.S.L. and 0.03% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, pH 6.5. Wet mycelia (about 5 g) of *Asp. sydowi* were inoculated in each 100 ml of medium containing 20% mustard extract, 0.1% yeast extract, 0.2% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.03% NaNO_3 , 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% glucose, pH 6.5. Total activities were measured by the liberation of glucose as described in the text.

○, Enzymatic activity in mycelia of *Asp. niger*; ●, Enzymatic activity in mycelia of *Asp. sydowi*.

With *Asp. niger* AKU 3302, the enzyme was produced in considerable amounts, mostly on the first and second days, without adjusting the pH range. *Asp. sydowi* also produces myrosinase, but it requires a long period for maximum production (Fig. 3). Some other strains of *Asp. niger* group tested, besides AKU 3302, produced the enzyme (Table II). *Asp. niger* did not produce the enzyme without inducer (mustard extract or sinigrin) (Table III), as with *Asp. sydowi*. The optimum concentration of mustard extract was 10% for *Asp. niger* and 20% for *Asp. sydowi*.¹⁾

Isothiocyanates (mustard oil) appear to be

very powerful fungicides.⁸⁾ Since allylthiocyanate is a product of sinigrin hydrolysis, there was a possibility that it might inhibit growth. *Asp. sydowi* probably required a higher concentration of mustard extract because of the longer cultivation period needed.¹⁾

It is pretty interesting that ascorbic acid which is a specific activator for plant myrosinase^{8,9)} is a good carbon source for fungous myrosinase production.

The intracellular myrosinase was very unstable, and was considerably inactivated during dialysis and salting out with ammonium sulfate. Concerning microbial enzymes, we often find stable enzymes in different strains of the same species. However, in the case of *Asp. niger*, enzymes from all the strains tested were unstable.

Addition of 2-mercaptoethanol, containing ascorbic acid to the enzyme solution was effective for stabilizing the enzyme. This may be related to the results of Table IV, which shows that ascorbic acid was an excellent carbon source.

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