General Characteristics of the Intracellular Myrosinase from *Aspergillus niger*[†]

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The enzymatic properties of intracellular myrosinase produced by *Aspergillus niger* AKU 3302 were investigated. Maximum activity occurred at pH 6.2, and the enzyme was stable in a pH range of 7.6 to 8.0 at 5°C for 24 hr. Optimum temperature was about 34°C. Enzyme activity was stimulated by copper (I), (II), manganese (II) and cobalt (II) and was inhibited by mercury (II) and stannous (II) ions. However, metal complexing agents and DFP had little effect, while PCMB was a strong inhibitor. In contrast to plant myrosinase, this enzyme was neither activated nor inhibited by L-ascorbic acid. Glucosides and δ -gluconolactone inhibited enzyme activity but sugars were ineffective. The K_m value for sinigrin was 3.3×10^{-3} M and that for *p*-nitrophenyl β -glucoside was 1.5×10^{-3} M. The relation between fungous myrosinases and β -glucosidase is discussed in comparison to plant myrosinase.

In a previous paper,¹⁾ *Aspergillus niger* AKU 3302, which produces myrosinase, was found through a screening test and its culture conditions were determined. The instability of this intracellular myrosinase was also reported.

There are several papers on the properties of plant myrosinase^{2~4)} and on extracellular myrosinase^{5,6)} produced by *Aspergillus sydowi* but the nature of intracellular myrosinase from microorganisms is not yet undetermined. There is only the report by Reese *et al.*⁶⁾ who pointed out the existence of intracellular myrosinase in the mycelia of fungus.

In this paper, we describe the general characteristics of the intracellular myrosinase produced by *Asp. niger* and compare them with characteristics of plant and extracellular myrosinases. The relationship of fungous and plant myrosinases to β -glucosidases is also discussed.

MATERIALS AND METHODS

Strain. *Aspergillus niger* AKU 3302 was kindly supplied by the Faculty of Agriculture of Kyoto University and was used as the enzyme source.

Culture. The strain was inoculated in a pre-culture medium (1.0 liter) 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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) at pH 6.5. Incubation was carried out at 29°C for 2 days under reciprocal shaking. The pre-culture was then inoculated into a 40-liter jar fermentor containing 30 liter of medium. The culture medium contained 10% mustard extract,¹⁾ 0.1% L-ascorbic acid, 0.1% C.S.L. and 0.03% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ in tap water at pH 7.0. Incubation was carried out at 29°C for 36 hr with aeration.

Enzyme preparation. The grown mycelia were harvested in a basket centrifuge, and washed twice with water. The collected mycelia were ground with sea sand in 0.1 M sodium phosphate buffer, pH 7.8, containing 0.01 M 2-mercaptoethanol and 0.001 M L-ascorbic acid after which the whole was centrifuged. The supernatant was used as the crude enzyme preparation.

Substrate. Sinigrin was prepared from yellow mustard seed^{2,7)} and was used as the substrate of fungous myrosinase. *p*-Nitrophenyl β -glucoside was of an analytical grade.

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Abbreviations: *p*-NPG, *p*-Nitrophenyl β -glucoside; ASA, L-Ascorbic acid; C.S.L., Corn Steep Liquor; DFP, Diisopropylfluorophosphate; PCMB, *p*-Chloromercuribenzoate; EDTA, Ethylenediaminetetraacetic Acid.

Assay of sinigrin hydrolysis. The assay mixture contained 2.5 μ moles of sinigrin and enzyme in a total volume of 1 ml. Enzymatic activities were determined by measuring liberated glucose and by titrating liberated sulfate at pH 6.2 and 34°C for 20 min, as described previously.^{2,5)} The reaction was conducted under conditions regarded to be those for zero order reactions.

Assay of *p*-NPG hydrolysis. The assay mixture contained 1 μ mole of *p*-NPG 0.1 μ mole of phosphate buffer, pH 6.2 and the enzyme in a total volume of 1 ml. The reaction was carried out at 34°C for 10 min under conditions regarded to be those for zero order reactions. After the reaction was over, 5 ml of 8% aqueous dipotassium hydrogen phosphate solution was added to the reaction mixture. Liberated *p*-nitrophenol was measured colorimetrically at 400 m μ using a Shimadzu Model QV-50 spectrophotometer.

Effects of various reagents. Reagents and enzyme were incubated at pH 6.2 and 34°C for 20 min before each experiment. Enzymatic reactions were initiated by the addition of substrate.

Assay of protein. Protein concentrations were determined by the method of Lowry *et al.*⁸⁾

RESULTS

Partial purification of the fungous myrosinase

Chromatography on DEAE-Sephadex. The crude enzyme solution (500 ml, total protein 1050 mg) was dialyzed against 0.01 M phosphate buffer containing 0.01 M 2-mercaptoethanol and 0.001 M ascorbic acid, pH 7.8, and then was charged on a DEAE-Sephadex column (4.5 $\times$ 23 cm) which was equilibrated with the same buffer. Elution of protein was performed with a linear gradient of 0.05 to 0.5 M sodium chloride in the same buffer. Myrosinase activity could not be separated completely from β -glucosidase activity (Fig. 1). The myrosinase in this step, however, was almost inactivated during dialysis and salting out with ammonium sulfate and was protected neither by the stabilizer (2-mercaptoethanol and ascorbic acid) of the crude preparation nor by any of reagents tested. But, the enzyme was only stable during concentrating with polyethylene glycol #6,000, further purification was done by an isoelectric focusing. Fractions indicated by the horizontal arrow

FIG. 1. Chromatogram of Fungous Myrosinase on a DEAE-Sephadex Column.

(○) Myrosinase activity and (●) β -Glucosidase activity (---) NaCl concentration.

FIG. 2. Isoelectrofocusing of the Partial Purified Enzyme.

The sample solution was isoelectrically focused on the LKB Column containing carrier ampholyte with a pH range of 4 to 6.

(○) Sinigrin hydrolysis, (●) *p*-NPG hydrolysis, (—) pH and (---) O.D. at 280 m μ .

in Fig. 2 were pooled and concentrated by polyethylene glycol.

Isoelectric focusing. Isoelectric focusing was carried out by the method of Vesterberg and Svensson,⁹⁾ using the LKB column (110 ml) containing carrier ampholyte and glycerol. The pH range of the ampholyte used was from 4 to 6. Ampholyte in the column amounted to 1 g. The anode compartment contained glycerol (60% v/v), to which 0.2 ml of phosphoric acid was added. The enzyme concentrated (50 mg protein,

TABLE I. SUMMARY OF PARTIAL PURIFICATION OF FUNGOUS MYROSINASE

Treatment	Volume (ml)	Activity (μ moles/min/ml)		Specific activity (μ moles/min/mg)		Protein (mg/ml)
		Sinigrin hydrolysis	<i>p</i> -NPG hydrolysis	Sinigrin hydrolysis	<i>p</i> -NPG hydrolysis	
Crude enzyme	500	0.291	0.447	0.139	0.213	2.10
Dialysate	510	0.270	0.391	0.134	0.195	2.02
Chromatography on DEAE-Sephadex	140	0.336	0.254	0.84	0.635	0.4
Concentration with Polyethylene glycol	20	1.68	1.429	0.672	0.572	2.5
Isoelectric focusing	6	0.543	0.164	1.916	0.58	0.283

about 20 ml) was applied to the column. The column was controlled at 4°C by circulating chilled water. The run was performed with a maximum load of 0.5 W, implying a final voltage of approximately 350 V. After focusing for 72 hr, fractions of each 2 ml were removed from the column and their enzyme activities were determined. In Fig. 2, typical isoelectrofocusing pattern is shown. There were some β -glucosidase activities for *p*-NPG besides myrosinase. The pI value of the myrosinase was about pH 4.8, the same as plant myrosinase. Fractions indicated by the horizontal arrow were pooled and used in the following experiments. A summary of the partial purification steps of the enzyme is shown in Table I. It also shows that fungus myrosinase is remarkably unstable.

General characteristics of the fungus myrosinase. Figures 3, 4, 5 and 6 show the pH-activity, pH-stability, temperature-stability and optimum-temperature curves, respectively. Optimum pH was about pH 6.2. The enzyme was most stable at pH 7.8 and at 5°C. Optimum temperature was at 34°C.

Effects of various reagents. Copper (I), (II), manganese (II) and cobalt (II) ions stimulated enzyme activity at 10^{-3} M; whereas, mercury (II) and stannous (II) ions inhibited it (Table II). PCMB was a strong inhibitor (Table III). DFP, iodoacetate and metal complexing agents showed little effect.

Plant myrosinase²⁾ is specifically activated by ascorbic acid (10^{-3} M), while, intracellular

FIG. 3. pH-Activity Curve of Fungous Myrosinase. 0.2 M Sodium phosphate buffer (pH 6.0~9.0) and 0.2 M acetate buffer (pH 5.0) were used.

FIG. 4. pH-Stability Curve of Fungous Myrosinase. The sample solution was incubated in 0.05 M sodium phosphate buffer (pH 6~9) and acetate buffer (pH 5.0) for 24 hr at 5°C. Reactions were carried out at pH 6.2 and 34°C.

FIG. 5. Heat-stability Curve of Fungous Myrosinase. The sample solution was incubated in 0.1 M phosphate buffer, pH 7.8 for 20 min at each temperature before measurement of enzyme activity.

myrosinase was neither activated nor inhibited by L-ascorbate.

δ -Gluconolactone, a specific inhibitor of β -glucosidase,¹⁰⁾ inhibited enzyme activity.

Effects of sugars and glucosides. Sugar and glucoside effects on intracellular myrosinase were tested (Table IV). None of the sugars tested had any effect, but glucosides inhibited activity by about 20~30%.

TABLE II. EFFECT OF INORGANIC SALTS ON FUNGOUS MYROSINASE

Reactions were carried out at pH 6.2 and 34°C. All inorganic salts were pre-incubated at 10^{-3} M.

Inorganic salts (10^{-3} M)	Relative activity (%)
None	100
KCl	95
LiCl	99
CuCl	140
CuCl ₂	132
MnCl ₂	170
CoCl ₂	186
MgCl ₂	77
ZnCl ₂	72
SnCl ₂	34
HgCl ₂	5
FeCl ₂	90
FeCl ₃	74

TABLE III. EFFECT OF VARIOUS REAGENTS ON FUNGOUS MYROSINASE

PCMB was pre-incubated at 6×10^{-5} M; others, at 10^{-3} M.

	Conc. (M)	Relative activity (%)
None		100
PCMB	6×10^{-5}	9
Iodoacetate	10^{-3}	80
EDTA	10^{-3}	80
<i>o</i> -Phenanthroline	10^{-3}	73
Ascorbic acid	10^{-3}	98
KI	10^{-3}	91
NaBr	10^{-3}	84
DFP	10^{-3}	82
δ -Gluconolactone	10^{-3}	38

TABLE IV. EFFECT OF VARIOUS SUGARS AND GLUCOSIDES ON FUNGOUS MYROSINASE

Reactions were carried out at pH 6.2 and 34°C. Enzyme activity was measured by the liberation of sulfate. Amygdalin and *p*-NPG were pre-incubated at 2×10^{-2} M and 5×10^{-2} M, respectively; others at 10^{-1} M.

Sugar or glucoside (0.1 M)	Relative activity (%)	
None	100	
Glucose	104	
Galactose	103	
Maltose	101	
Fructose	107	
Xylose	105	
Salicin	85	
Arbutin	79	
Amygdalin	2×10^{-2} M	86
<i>p</i> -NPG	5×10^{-2} M	106

Kinetic studies. Figure 7 shows the Lineweaver-Burk plot for intracellular myrosinase. *Km* value for sinigrin was calculated to be 3.3×10^{-3} M.

In order to prove that *p*-NPG was hydrolyzed by catalytic action of the fungous myrosinase itself, as described by Levvy and Mash,¹¹⁾ the inhibitory effect of the specific substrate, sinigrin, on *p*-NPG hydrolysis was tested. Results are shown in Fig. 8. It is evident from this figure that *p*-NPG hydrolysis was inhibited competitively by sinigrin. The *Km* value for *p*-NPG and *Ki* value for sinigrin were calculated to be 1.5×10^{-3} M and 3.8×10^{-3} M, respectively. This *Ki* value is in good

TABLE V. COMPARISON OF SOME PROPERTIES OF MYROSINASES FROM VARIOUS ORIGINS

	<i>Asp. niger</i>	<i>Asp. sydowi</i>	Plant
pH-activity	6.2	7	5~7
pH-stability	7.8	6~9	4~9
Heat-stability	25°C	45°C	55°C
PCMB	inhibit	no effect	inhibit
Ascorbic acid	no effect	no effect	activate
Metal	activate (Cu ⁺ , Cu ²⁺ , Mn ²⁺ , Co ²⁺)	activate (Co ²⁺)	—
	inhibit (Hg ²⁺ , Sn ²⁺)	inhibit (Hg ²⁺ , Fe ²⁺)	inhibit (Hg ²⁺ , Cu ²⁺)
<i>K_m</i> for Sinigrin	3.3×10^{-3} M	3.6×10^{-3} M	1.8×10^{-4} M
<i>K_i</i> for Sinigrin	3.8×10^{-3} M	3.8×10^{-3} M	2.0×10^{-4} M
<i>K_m</i> for <i>p</i> -NPG	1.5×10^{-3} M	1.0×10^{-4} M	2.0×10^{-3} M

FIG. 6. Optimum-temperature Curve of Fungous Myrosinase.

Reactions were carried out at pH 6.2 for 20 min.

agreement with the *K_m* value for sinigrin (3.3×10^{-3} M) of the enzyme.

DISCUSSION

General characteristics of intracellular myrosinase produced by *Aspergillus niger* AKU 3302 were investigated. There were some β -glucosidase activities for *p*-NPG in the mycelia of *Asp. niger*, besides myrosinase. As described previously,¹⁾ the myrosinase produced by *Asp. niger* was remarkably un-

FIG. 7. Reciprocal Plot of Sinigrin Hydrolysis.

stable and became more so after chromatography on DEAE-Sephadex. This myrosinase was not protected by stabilizing reagents for the crude preparation.

Some properties of *Asp. niger*, *Asp. sydowi* and plant myrosinases were compared (Table V). *Asp. niger* myrosinase was less stable than the others to heat, acid and alkali, and to chemicals. It was strongly inhibited by PCMB. The *Asp. sydowi* myrosinase prepared by the authors was not inhibited by PCMB but its inhibition has been reported by Reese *et al.*⁶⁾

Asp. niger myrosinase was activated by cobalt (II) and manganese (II) and was inhibited by mercury (II). However, metal com-

FIG. 8. Inhibitory Effect of Sinigrin on *p*-NPG Hydrolysis.

Reactions were carried out in the presence (●) and absence (○) of 2.5 mM sinigrin at pH 6.2 and 34°C.

plexing agents (EDTA and *o*-phenanthroline) only slightly inhibited the activity. Thus, the role of metal ions to the enzyme is still unsolved.

K_m value for the sinigrin of fungous myrosinases are considered to be larger than that for *p*-NPG, *i.e.* fungous myrosinases are thought to have a greater affinity for *p*-NPG, whereas plant myrosinase has a greater affinity for sinigrin than for *p*-NPG. Thus, *Asp. niger* myrosinase is also thought to be a β -glucosidase, which has high affinity for mustard oil glucoside. Pigman,¹²⁾ Nagashima and Uchiyama,^{13,14)} and the authors⁴⁾ have reported that plant myrosinase is a member of the β -glucosidase. The above

results support this conclusion. Plant myrosinase is highly specified for mustard oil glucosides, whereas fungous myrosinases are less specific than the plant enzyme. Even so, fungous myrosinases have higher affinities for mustard oil glucosides than for the usual β -glucosidases. This conclusion is supported by the fact that fungous myrosinase was produced only in the presence of mustard extract.

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