Purification and General Characteristics of Bacterial Myrosinase Produced by *Enterobacter cloacae*[†]

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Myrosinase in cell-free extract of *Enterobacter cloacae*, no. 506, was purified about 1,000 fold by precipitation with ammonium sulfate, chromatography on CM-Sephadex and gel-filtration on Sephadex G-200 and Sephadex G-100. The enzyme was shown to be homogeneous by chromatography and by ultracentrifuge. Molecular weight obtained by gel-filtration was 61,000 and the sedimentation coefficient was 4.5 S. Maximum activity occurred at pH 6.8. The enzyme was stable in a pH range of 5.0 to 7.0 at temperature below 40°C and for 24 hr. Copper(I) and (II), mercury (II) and ferrous(II) ions strongly inhibited the activity. Sulfhydryl reagents had little effect but EDTA was a strong inhibitor. In contrast to plant myrosinase, this enzyme was inhibited by L-ascorbic acid. Many glucosides and sugars inhibited the enzyme. The relation between bacterial myrosinase and β -glucosidase is discussed in comparison to plant and fungous myrosinases. Some comparative properties of bacterial, fungous and plant myrosinases are discussed.

In our previous study,¹⁾ *Enterobacter cloacae*, no. 506, which produced myrosinase, was found in a screening test and its culture conditions were determined.

The properties of plant and fungous myrosinases are well documented, but the nature of bacterial myrosinase is not yet known. Oginsky *et al.*²⁾ have reported on its existence and some properties using crude preparations of bacterial myrosinase.

In this paper, we report on the purification and the general characteristics of bacterial myrosinase produced by *Enterobacter cloacae* and compared these characteristics with previous observations on plant^{3~5)} and fungous myrosinases.^{6,7)} The relationship of bacterial, fungous and plant myrosinases to β -glucosidases is also discussed.

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Abbreviation: *p*-NPG, *p*-nitrophenyl β -glucoside; ASA, L-ascorbic acid; DFP, diisopropylfluorophosphate; PCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetic acid.

MATERIALS AND METHODS

Materials. All chemicals were obtained from commercial sources and were used without further purification.

Cultivation. The cultivation was carried out as described previously.¹⁾

Enzyme preparation. The cell paste (250 g) was suspended in 0.2 M phosphate buffer, pH 7.0, to give a suspension of about 1 g per 9 ml. The suspension was subjected to the action of a Kaijo Denki ultrasonic oscillator (20 kc) for 90 min. Cells and debris were removed by centrifugation at 10,000 rpm for 20 min. The resultant supernatant was used for the crude enzyme preparation.

Enzyme assay

1) **Thioglucosidase and sulfatase activities.** Enzymatic activities were determined as thioglucosidase and sulfatase activities. Both reactions were carried out in a system containing 2.5 μ moles of sinigrin and 0.1 μ mole of phosphate buffer, pH 7.0, in a total volume of 1 ml. In the assay system for sulfatase activity, phosphate buffer was omitted. The reactions were carried out at 37°C and pH 7.0, unless otherwise mentioned. Thioglucosidase activity and sulfatase activity were represented as the liberation of glucose and sulfate, respectively. The reactions were conducted under conditions regarded to be zero order reactions.

Glucose was determined by Sumner's dinitrosalicylic

acid method, with the previously described modification.⁶¹ The data obtained were corrected by multiplying by the factor 1.25 to eliminate the color development caused by the substrate itself.

Sulfate was determined by titration with 0.01 N NaOH, using a recording pH-stat, Radiometer Model SBR2/SBU/TTT1 Autotitrator at pH 7.0 under nitrogen gas stream.

2) *β -Glucosidase activity.* The assay mixture contained 1 μ mole of *p*-NPG, 0.1 μ mole of phosphate buffer, pH 7.0, and the enzyme in a total volume of 1 ml. The reaction was carried out at 37°C for 10 min. After the reaction was over, 5 ml of 8% K_2HPO_4 was added to the reaction mixture. Liberated *p*-nitrophenol was measured by colorimetry at 400 nm.

Assay of protein. Protein concentration was determined by the method of Lowry *et al.*⁸¹

Estimation of molecular weight. The molecular weight of the enzyme was estimated by Andrew's method⁹¹ using a Sephadex G-100 column equilibrated with 0.01 M phosphate buffer containing 0.1 M KCl at pH 6.4. Bovine serum albumin (M.W. 67000), ovalbumin (M.W. 45000), chymotrypsinogen (M.W. 25000) and cytochrome C (M.W. 12400) were used as standard proteins.

Calculation of sedimentation coefficient. Sedimentation studies were carried out with a HITACHI model UCA-1A ultracentrifuge. All runs were performed at 60,000 rpm and at 20°C in a double sector cell. The $s_{20,w}$ was calculated from series of sedimentation runs according to the conventional method.¹⁰¹

RESULTS

The procedure for the purification of bacterial myrosinase is shown in Fig. 1. The crude preparation, as described in the methods,

FIG. 1. Purification Procedure of Bacterial Myrosinase.

precipitated at 0.3 to 0.8 ammonium sulfate saturation was dialyzed against 0.01 M potassium phosphate buffer, pH 6.5, and subjected to CM-Sephadex C-50 column (8 × 40 cm) that

FIG. 2. Rechromatogram of Bacterial Myrosinase on Sephadex G-100.

(●), Myrosinase activity; (---), β -glucosidase activity; (○), protein.

TABLE I. SUMMARY DATA ON PURIFICATION OF BACTERIAL MYROSINASE

	Volume (ml)	Activity (μ moles/min/ml)	Specific activity (μ moles/min/mg)	Protein (mg)
Crude preparation	2100	747.6	0.0116	41000
Chromatography on CM-Sephadex	250	410	0.59	700
Gel-filtration on Sephadex G-200	80	280	2.8	100
Gel-filtration on Sephadex G-100	40	162	5.9	27.6
Rechromatography on Sephadex G-100	35	175	11.5	15

FIG. 3. The Sedimentation Pattern of the Purified Enzyme.

Condition: protein, 5 mg/ml; solvent, 0.01 M potassium phosphate buffer (pH 6.5) containing 0.1 M KCl; temperature, 20°C; speed, 60,000 rpm.

was equilibrated with the same buffer. Adsorbed protein was eluted with a linear gradient of 0.1 to 0.3 M sodium chloride in the same buffer. The active fractions were combined and precipitated by 0.8 satn. of ammonium sulfate. The precipitate obtained by centrifugation was dissolved in 15 ml of 0.01 M potassium phosphate buffer, pH 6.5, and subjected to Sephadex G-200 gel filtration. Sephadex G-200 was packed in a column (5×80 cm) and equilibrated with 0.01 M potassium phosphate buffer, pH 6.5. The eluted active fractions were pooled and precipitated by 0.8 satn. of ammonium sulfate. The precipitate obtained by centrifugation was dissolved in 5 ml of 0.01 M potassium phosphate buffer, pH 6.5, and subjected to Sephadex G-100 column (2.6×90 cm). Finally, the enzyme solution (3 ml, 27 mg of protein) was purified by rechromatography on Sephadex G-100 column (2.6×90 cm). As shown in Fig. 2, a chromatographically homogenous pattern of the enzyme was obtained. This filtrate was used throughout the following experiments. The summary data on enzyme purification is shown in Table I.

The enzyme was purified approximately to 1000-fold compared to the crude preparation.

Homogeneity of bacterial myrosinase

The analysis of purified enzyme by ultracentrifugation showed a single and symmetrical pattern (Fig. 3). The sedimentation coefficient, s_{w}^{20} , was calculated to be 4.5 S from sedimentation velocity at 60,000 rpm.

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FIG. 4. Determination of Molecular Weight of Bacterial Myrosinase by Gel-Filtration on Sephadex G-100.

Estimation of the molecular weight

Molecular weight was determined by gel-filtration through Sephadex G-100. Figure 4 shows the plots of the elution volumes versus the logarithms of the known molecular weight of standard proteins. The molecular weight of the enzyme was estimated to be 61,000.

General characteristics of bacterial myrosinase

Figures 5, 6 and 7 show pH-activity, pH-

stability and temperature-stability curves, respectively. Optimum pH was about 6.8. The enzyme was stable in the pH range from 5 to 7 and at temperatures below 40°C.

Figures 8 and 9 show the Lineweaver-Burk plot of the enzyme. The K_m values for sinigrin and *p*-NPG were calculated to be 3.7×10^{-4} M and 7.1×10^{-4} M, respectively.

FIG. 5. pH-Activity Curve of Bacterial Myrosinase. The enzyme activity was measured by the liberation of sulfate.

FIG. 6. pH-Stability Curve of Bacterial Myrosinase. The enzyme (15 μ g) was incubated with 0.01 μ mole of acetate (pH 4.5~6), phosphate (pH 6~8) and Tris-HCl (pH 8~) buffer in 0.3 ml for 24 hr at 20°C. The enzyme was then assayed by addition of 0.5 ml of 0.2 M phosphate buffer, pH 6.8, and 0.2 ml of substrate. Enzyme activity was measured by the liberation of glucose.

FIG. 7. Heat-Stability Curve of Bacterial Myrosinase. The sample solution was preincubated in 0.2 M phosphate buffer, pH 6.8, for 30 min at each temperature before measurement of enzyme activity.

FIG. 8. Reciprocal Plot of Sinigrin Hydrolysis. Enzyme activity was measured by the liberation of sulfate.

Effect of various reagents

Sulfhydryl reagents such as pCMB and iodoacetate showed no inhibitory effect. The activity was considerably inhibited by the chelating agent EDTA but not inhibited by *o*-phenanthroline. δ -Gluconolactone, which is a specific inhibitor on β -glucosidase, showed an inhibitory effect on the purified enzyme. L-Ascorbic acid, which is the specific activator on plant myrosinase, showed a remarkable inhibitory effect on this enzyme (Table II).

FIG. 9. Reciprocal Plot of *p*-NPG Hydrolysis.

TABLE II. EFFECT OF VARIOUS REAGENTS ON BACTERIAL MYROSINASE

The enzyme solutions were preincubated with each reagent for 30 min at 37°C. The enzyme (15 μg) was incubated with inhibitor and 0.1 μmoles of phosphate buffer (pH 6.8) in 0.8 ml, then 0.2 ml of substrate was added for activity assay. Enzyme activity was measured by the liberation of glucose.

Reagent	Conc.(M)	Relative activity(%)
None		100
PCMB	10 ⁻⁴	97
Iodoacetate	10 ⁻³	109
DFP	10 ⁻³	101
EDTA	10 ⁻⁴	34
EDTA	10 ⁻³	25
<i>o</i> -Phenanthroline	10 ⁻³	92
Dithiothreitol	10 ⁻⁴	97
Mercaptoethanol	10 ⁻³	130
δ-Gluconolactone	10 ⁻²	65
Ascorbic acid	10 ⁻⁴	12

Many metal ions inhibited enzyme activity to some extent. Among these, Cu⁺, Cu²⁺, Hg²⁺ and Fe²⁺ strongly inhibited the activity (Table III).

Effect of various glucosides and sugars

Bacterial myrosinase has β-glucosidase activity (Fig. 2) as well as myrosinase activity, like plant and fungous myrosinases. In order to clarify the relationship between myrosinase activity and β-glucosidase activity of bacterial myrosinase, the effect of glucosides and sugars

TABLE III. EFFECT OF INORGANIC SALTS ON BACTERIAL MYROSINASE

The enzyme solutions were preincubated with each salt for 30 min at 37°C. The enzyme (15 μg) was incubated with salt and 0.1 μmoles of phosphate buffer (pH 6.8) in 0.8 ml, then 0.2 ml of substrate was added for activity assay. Enzyme activity was measured by the liberation of glucose.

Salt	Conc. (M)	Relative activity (%)
None		100
CoCl ₂	10 ⁻³	73
ZnCl ₂	10 ⁻³	55
CaCl ₂	10 ⁻³	86
MgCl ₂	10 ⁻³	78
SrCl ₂	10 ⁻³	79
CuCl	10 ⁻³	44
CuCl ₂	10 ⁻³	22
HgCl ₂	10 ⁻³	13
NiCl ₂	10 ⁻³	70
FeCl ₂	10 ⁻³	32
FeCl ₃	10 ⁻³	82
LiCl	10 ⁻³	120
SnCl ₂	10 ⁻³	103
MnCl ₂	10 ⁻³	93
NaCl	10 ⁻³	95

was tested. All glucosides tested showed an inhibitory effect (Table IV) with salicin, amygdalin, arbutin and *p*-NPG showing particularly strong inhibitory effects. The effect of various sugars are shown in Table V. All sugars tested showed inhibitory effects.

The inhibitory effect of salicin on sinigrin hydrolysis is demonstrated as Lineweaver-Burk plot in Fig. 10, plotted in the absence

TABLE IV. EFFECT OF VARIOUS GLUCOSIDES ON BACTERIAL MYROSINASE

The reactions were carried out in the presence of 0.1 M glucoside at pH 6.8 and 37°C. Enzyme activity was measured by the liberation of sulfate.

Glucoside (0.1 M)	Relative activity (%)
None	100
Salicin	23
Amygdalin	13
Arbutin	4
α-Me-glucoside	87
β-Me-glucoside	59
β-Phenylglucoside	34
<i>p</i> -Nitrophenyl β-glucoside	12

TABLE V. EFFECT OF VARIOUS SUGARS ON BACTERIAL MYROSINASE

The reactions were carried out in the presence of 0.1 M sugar at pH 6.8 and 37°C. Enzyme activity was measured by the liberation of sulfate.

Sugar (0.1 M)	Relative activity (%)
None	100
Glucose	32
Fructose	75
Galactose	70
Mannose	63
Sucrose	77
Maltose	80
Xylose	94
Sorbitol	71

FIG. 10. Inhibitory Effect of Salicin on Sinigrin Hydrolysis.

Enzyme activity was measured by the liberation of sulfate. Reactions were carried out in the presence (●) and absence (○) of 4 mM salicin at pH 7.0 and 37°C.

and presence of salicin. The inhibition by salicin is competitive. The K_i value for salicin was calculated to be 6.6×10^{-4} M.

Substrate specificity

The hydrolytic activity on various glucosides was tested (Table VI). The bacterial myrosinase hydrolyzed *p*-NPG besides sinigrin.

Effect of sugar and glucosides on *p*-NPG hydrolysis

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TABLE VI. SUBSTRATE SPECIFICITY OF BACTERIAL MYROSINASE

The hydrolytic rates were determined at 4 mM for each substrate. Reactions were carried out at pH 6.8 and 37°C. Enzyme activity was measured by the liberation of glucose. *p*-NPG hydrolysis was performed as described in the text.

Substrate	Hydrolytic rate (μmoles/min/ml)
Sinigrin	1.073
Salicin	0
Arbutin	0
α-Methylglucoside	0
β-Methylglucoside	0
β-Phenylglucoside	0
<i>p</i> -Nitrophenyl β-glucoside	0.616

FIG. 11. Inhibitory Effect of Sugar and Glucosides on *p*-NPG Hydrolysis.

Reactions were carried out in the presence of 2.5 mM sinigrin (Δ), of 4 mM salicin (●), of 0.1 M glucose (---) and absence (○) of these at pH 7.0 and 37°C.

ed. As shown in Fig. 11, glucose, sinigrin and salicin showed competitive inhibition. The K_i values for glucose, sinigrin and salicin were calculated to be 1.2×10^{-4} M, 3×10^{-4} M and 5.5×10^{-4} M, respectively.

DISCUSSION

General characteristics of bacterial myrosinase produced by *Enterobacter cloacae*, no. 506, were investigated. Some properties of bacterial, fungus and plant myrosinases were

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

	Plant ^{3,5,14)}	<i>Asp. niger</i> ⁷⁾	<i>Asp. sydowi</i> ⁶⁾	<i>Enterobacter cloacae</i>
pH-activity	5~7	6.2	7	6.8
pH-stability	4~9	7.8	6~9	5~7
Heat stability	55°C	45°C	45°C	40°C
PCMB	inhibit	inhibit	no effect	no effect
Ascorbic acid	activate	no effect	no effect	inhibit
EDTA	no effect	no effect	no effect	inhibit
Metal Activation	none	Co ²⁺ , Cu ²⁺ , Mn ²⁺	Co ²⁺	none
Inhibition	Cu ²⁺ , Hg ²⁺	Sn ²⁺ , Hg ²⁺	Fe ²⁺ , Hg ²⁺	Cu ²⁺ , Hg ²⁺
Molecular weight	120,000~150,000	90,000	120,000	61,000
<i>Km</i> for sinigrin	$1.8 \times 10^{-4}\text{M}$	$3.3 \times 10^{-3}\text{M}$	$3.6 \times 10^{-3}\text{M}$	$3.7 \times 10^{-4}\text{M}$
<i>Km</i> for <i>p</i> -NPG	$2.0 \times 10^{-3}\text{M}$	$1.5 \times 10^{-3}\text{M}$	$1.0 \times 10^{-4}\text{M}$	$7.1 \times 10^{-4}\text{M}$
<i>Ki</i> for sinigrin (<i>p</i> -NPG hydrolysis)	$2.0 \times 10^{-4}\text{M}$	$3.8 \times 10^{-3}\text{M}$	$3.8 \times 10^{-3}\text{M}$	$3.0 \times 10^{-4}\text{M}$
<i>Ki</i> for salicin (Sinigrin hydrolysis)	$1.8 \times 10^{-1}\text{M}$		$1.6 \times 10^{-1}\text{M}$	$6.6 \times 10^{-4}\text{M}$

compared (Table VII). The effect of pH and heat on bacterial enzyme is rather stable like that of plant enzyme and fungous enzyme from *Aspergillus sydowi*. However, PCMB which inhibited the fungous and plant myrosinase showed no inhibitory effect on bacterial myrosinase. EDTA which showed no inhibitory effect on plant and fungous myrosinase inhibited bacterial myrosinase. Metal ion Hg²⁺ inhibited all enzymes. L-Ascorbic acid which is the specific activator on plant enzyme inhibited the bacterial enzyme. Plant myrosinase indicated a rather large molecular weight of 150,000 while bacterial enzyme had the smallest weight of the four myrosinases.

As in fungous and plant myrosinase, *Ki* value for sinigrin in *p*-NPG hydrolysis is in good agreement with the *Km* value for sinigrin in sinigrin hydrolysis. This indicates that as in fungous and plant myrosinase, the hydrolysis of *p*-NPG take place on the same site as in the case of sinigrin. The *Km* values for sinigrin and *p*-NPG differ in bacterial myrosinase from fungous myrosinase as the ratio of *Km* values for sinigrin to *p*-NPG is in the order plant < bacteria < *Asp. niger* < *Asp. sydowi*. These findings suggest that bacterial myrosinase may be considered as β -glucosidase which has a high affinity for mustard oil

glucoside. Pigman,¹¹⁾ Nagashima and Uchiyama,^{12,13)} and the authors⁵⁾ have reported that plant myrosinase is a member of the β -glucosidase family. The results of the present study further support this conclusion. Plant myrosinase is highly specific for mustard oil glucosides whereas fungous myrosinases are less specific than plant enzyme, but the specificity of bacterial myrosinase is between plant and fungous enzyme and is thought to be rather similar to plant enzyme than to fungous enzyme. Even so, microbial myrosinases have high affinities for mustard oil glucosides than for the usual β -glucosidases. This conclusion is supported by the fact that microbial myrosinases were produced only in the presence of inducers such as sinigrin and mustard extract.

The different behaviours and characteristics of the four enzymes become an interesting factor. It is particularly noteworthy that ascorbic acid has a very prominent effect on each enzyme and further investigations are required to clarify the inhibitory effect by ascorbic acid on bacterial myrosinase.

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REFERENCES

- 1) N. Tani, M. Ohtsuru and T. Hata, *Agr. Biol. Chem.*, **38**, 1617 (1974).
 - 2) E. L. Oginsky, A. E. Stein and M. A. Green, *Proc. Soc. Exp. Med.*, **119**, 360 (1965).
 - 3) I. Tsuruo, M. Yoshida and T. Hata, *Agr. Biol. Chem.*, **31**, 18 (1967).
 - 4) I. Tsuruo and T. Hata, *ibid.*, **31**, 27 (1967).
 - 5) I. Tsuruo and T. Hata, *ibid.*, **32**, 1425 (1968).
 - 6) M. Ohtsuru, I. Tsuruo and T. Hata, *ibid.*, **33**, 1309 (1969).
 - 7) M. Ohtsuru and T. Hata, *ibid.*, **37**, 2543 (1973).
 - 8) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
 - 9) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
 - 10) H. K. Schachman, In "Methods in Enzymology," Vol. 4, ed. by S. P. Colowick and H. O. Kaplan, Academic Press, New York, 1957, p. 32.
 - 11) W. W. Pigman, *J. Res. Natl. Bur. Stand.*, **30**, 257 (1943); *Advances in Enzymology*, **4**, 41 (1944).
 - 12) Z. Nagashima and M. Uchiyama, *Nippon Nogekagaku Kaishi*, **33**, 1144 (1959).
 - 13) Z. Nagashima and M. Uchiyama, *Bull. Agr. Chem. Soc. Japan*, **23**, 555 (1959).
 - 14) M. Ohtsuru and T. Hata, *Agr. Biol. Chem.*, **36**, 2495 (1972).
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