STUDIES ON MYROSINASE

MASARU OHTSURU

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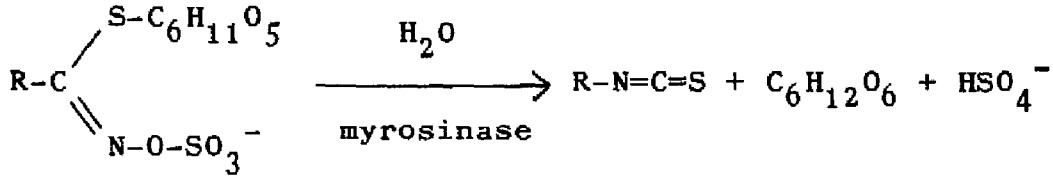
CONTENTS

ABBREVIATIONS

ASA	L-Ascorbic Acid
p-NPG	p-Nitrophenyl β-glucoside
C.S.L.	Corn Steep Liquor
PCMB	p-Mercuribenzoate
DFP	Diisopropylfluorophosphate
EDTA	Ethylenediaminetetraacetic Acid
FDNB	Fluorodinitrobenzene
TNBS	Trinitrobenzenesulfonic Acid
CFQ	Monochlorotrifluoro-p-benzoquinone
NBS	N-Bromosuccinimide
SDS	Sodium dodecylsulfate
MNT	2-Methoxy-5-nitrotropone
DEP	Diethylpyrocarbonate
Ellman reagent	5-5'-Dithio-bis-(2-nitrobenzoic acid)
Koshland reagent	2-Hydroxy-5-nitrobenzyl bromide
Mustard Ext.	Extract of mustard seed (1.0 Kg) by 80% methanol (5.0 L) was concentrated to 700 ml.

INTRODUCTION

Myrosinases [thioglucoside (glucosinolate) glucohydrolase, EC 3.2.3.1] are the enzymes responsible for the hydrolysis of mustard oil glucosides, which have been found in plants (Cruciferae family)[1,2], fungi[3], bacteria[4] and mammals[5]. The action of myrosinase on the mustard oil compounds is the hydrolysis of glucose from the thioglucoside followed by Lossen rearrangement of the aglycon to give isothiocyanate and sulfate[6,7].

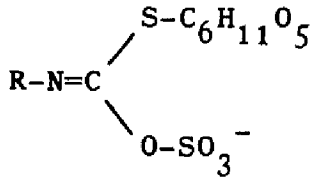


Sinigrin: R- = CH_2=CH-CH_2-

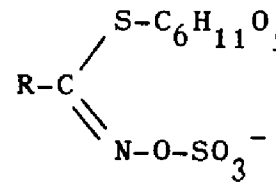
The myrosinase is responsible for the development of the flavor and pungency of many food products, such as mustard and horseradish, by its hydrolysis of the thioglucosides. Thus, myrosinase is a kind of the typical flavor enzymes and would become a model of the flavor development by enzymes. Further, the enzyme converts progoitrin which is a thioglucoside present in high concentration in the seeds of most Brassicaceous plants and rapeseed meal, into goitrin, a potent antithyroid compound[4]. Therefore, it also becomes necessary to clarify the characteristics of myrosinase in order to utilize the rapeseed meal for the feeding of livestock.

There had been some controversies concerning the nature of the myrosinase on the plant myrosinase.

Previously, Gadamer[8] assigned the structure (I) for mustard oil glucoside. Neuberg et al.[9] reported the separation of thioglucosidase and sulfatase which hydrolyzed thioglucoside linkage and sulfuric acid ester linkage of (I), respectively. Thereafter, it was generally believed[10] that the myrosinase in plants was a mixture of the thioglucosidase and the sulfatase. Ettlinger et al.[6,7], however, corrected the structure (I) to the structure (II) and suggested that the liberation of sulfate would occur by the Lossen rearrangement after the cleavage of the thioglucoside linkage. Nagashima et al.[11] also supported the mechanism of Lossen rearrangement, and concluded that the myrosinase was not a mixture of the two enzymes but a single β-thioglucosidase and the liberation of sulfate would occur nonenzymatically.



(I)



(II)

Virtanen et al.[12] found an enzymatic production of thiocyanate beside isothiocyanate from the mustard oil glucoside in some species of the plants exhibiting myrosinase activity. As a result, some uncertainty occurred in the theory of Lossen rearrangement of the glucoside. Therefore, Gaines et al.[13,14] carried out further investigation on the homogeneity of the myrosinase, and successfully separated thioglucosidase and sulfatase.

Furthermore, Ettlinger et al.[15] suggested that there may exist two additional enzymes, one of which is activated by L-ascorbic acid.

Tsuruo et al.[16] purified the myrosinase, using a TEAE-cellulose chromatography and showed that thioglucosidase and sulfatase activities were not distinguishable. So, they concluded the myrosinase is a single β-thioglucosidase. Later, Gaines and Howard[17] accepted the theory that the myrosinase is not a mixture of two enzymes but a single β-thioglucosidase.

Nagashima et al.[18] found that the plant myrosinase was activated strongly by L-ascorbic acid. Schwimmer[19] and Ettlinger et al.[15] investigated this phenomenon. The later concluded that ascorbic acid behaved as a reversibly dissociable base, closely connected with the nucleophilic group of thioglucosidase. Kojima et al.[20] studied the change of the reaction product of the enzymatic hydrolysis of mustard oil glucoside in the presence of ascorbic acid at low pH. Tsuruo and Hata[21] described that the oxidation-reduction reaction of ascorbic acid had nothing to do with the activation reaction of myrosinase. The presence of the effector site for ascorbic acid was presumed by the kinetic measurements and instabilization of the enzyme by ascorbate on heating.

But, the above conclusions have been derived from the investigations using the crude preparations. Thus, the author has investigated the purification, physical and chemical properties of the myrosinase from yellow mustard powder, and the activation mechanism of the myrosinase by ascorbate.

While, Reese et al.[3] reported that fungi (Aspergillus sydowi etc.) produced a myrosinase (Sinigrinase). Kojima et al.[22] reported some of its properties. Thereafter, Oginsky et al.[4] reported that the myrosinase activity existed in Paracolobactrum aerogenoides and in several other bacterial strains.

This study was performed to intensify the understanding on the myrosinase, which was stimulated by my belief that microorganisms would be a more economical source of the enzymes required for improvement of the flavor of processed foods and the utilized resources (for example, Rapeseed meal etc.) than those from higher plants.

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PART I

Studies on the Plant Myrosinase

Chapter 1

Chemical and Physical Properties

In the preceding works,[1~4] Tsuruo et al. reported various properties of plant myrosinase, which is a kind of β-thioglucosidase. Plant myrosinase is specifically activated by L-ascorbate[5] and its activation mechanism had been investigated by kinetic procedures. But, to discuss the activation mechanism of the plant myrosinase by L-ascorbate, it is necessary to obtain the detailed information about physico-chemical nature of the purified enzyme.

I had purified the plant myrosinase to investigate the activation mechanism of the enzyme by L-ascorbate. During the course of purification, four proteins which have myrosinase activity were separated. In this Chapter, purification procedures and physical and chemical properties of each enzyme are described.

MATERIAL AND METHODS

Material and chemicals. Mustard powder (Canada Oriental Mustard Seed) was purchased from Amari Koshin Shokuhin K. K. Sinigrin was obtained from Nakarai Chemicals Ltd. and used as the substrate.

Enzyme purification. The procedure of purification of plant myrosinase is shown in Fig. 1. The enzyme solution was prepared from mustard powder. The extract (100 liters) from mustard powder (40 kg) by 0.1 M sodium phosphate buffer containing 0.01 M 2-mercaptoethanol was fractionated by ammonium sulfate at pH 7.0. The fraction precipitated at 0.4 to 0.8 of ammonium sulfate saturation was dialyzed against 0.01 M sodium phosphate buffer, pH 7.0 and subjected to DEAE-Sephadex A-50 (6 l) column chromatography. The adsorbed protein was eluted batchwise with 10 liters of the same buffer containing 0.2 M sodium chloride. The eluted protein was precipitated at 90% saturation with ammonium sulfate and was dialyzed against the same buffer. The dialyzate was applied to DEAE-Sephadex column (5×10 cm). Elution of the protein was made with a linear gradient of 0 to 0.2 M sodium chloride in the same buffer (Fig. 2). Two separate peaks with myrosinase activity were eluted and are designated as F-I fraction and F-II fraction, respectively.

F-I and F-II fractions were rechromatographed on DEAE-Sephadex under the same condition (Fig. 2). After concentration of each fraction by ammonium sulfate, gel-filtration on Sephadex G-200 columns (2 l, 5×110 cm) were carried out. Then, each filtrate was respectively charged on CM-Sephadex column (4×20 cm), equilibrated with 0.01 M sodium acetate buffer, pH 5.0 and the adsorped protein was eluted by a linear gradient of 0 to 0.2 M sodium chloride (Fig. 3). Two peaks with myrosinase activity were separated from each fraction (F-I, F-II) and are named F-IA, F-IB, F-IIA and F-IIB, respectively. F-IA, F-IB and F-IIA fractions were rechromatographed on CM-Sephadex under the same condition. F-IIB was charged on DEAE-Sephadex column equilibrated with 0.01 M sodium phosphate buffer, pH 8.5 and eluted with a linear gradient of 0 to 0.2 M sodium chloride, because F-IIB fractions could not be purified by CM-Sephadex, pH 5.0. Finally, the enzymes were purified by gel-filtration on Sephadex G-200 column (2.5×90 cm) (Fig. 4).

Enzyme assay. The assay mixture contained 2.5 μmoles of substrate and enzyme in a total volume of 1 ml. Enzymatic activities were measured by the liberation of glucose.[6] The reactions were carried out under conditions regarded to be zero order reactions.

Assay of protein. Protein was determined by the method of Lowry *et al.*[7]

Determination of physical characteristics. Prior to each determination, the protein solutions were equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M KCl by dialysis overnight at 5°C.

Disc electrophoresis was done with a 7.5% polyacrylamide gel column at pH 7.0. Three μl of tracking dye (0.05% Bromphenol blue in water), 1 drop of glycerol, 5 μl of 2-mercaptoethanol, 50 μl of sodium phosphate buffer (0.1 M), and 20 μl of protein (200 μg) are mixed and of the mixture 5 to 10 μl were applied on the gels according to the procedure of Osborn[8] and Davis.[9] Electrophoresis was performed at a constant current of 2 mA per gel with the positive electrode in the lower chamber.

Isoelectric focusing was carried out by the method of Vesterberg and Svensson,[10] using LKB column (110 ml) containing carrier ampholine within a pH range of 3 to 10. Electrophoresis was performed with the potential gradient of 350 V (0.5 W) for 72 hr, maintaining column temperature at 5°C by circulating chilled water. After the electrophoretic run, the ampholine was fractionated to 2 ml, and the pH of each fraction was measured.

Sedimentation patterns of the preparation were followed with a Hitachi model UCA-1A ultracentrifuge at 51,200 rpm in a double sector cell or a single sector cell. Final protein concentration was determined by counting the Rayleigh fringe from interference pictures taken during sedimentation. All runs were made at 20°C. Analysis of photographs were made with a Nikon comparameter. The s_w^{20} was determined from a series of sedimentation runs at pH 7.0 and pH 5.0. Sedimentation equilibrium studies were carried out at a speed below 9,620 rpm in a three sample cell. Slopes of linear plots of the logarithmic fringe displacement against the radial distance squared were analyzed according to conventional sedimentation equilibrium method[11] to calculate the apparent weight-arange molecular weight.

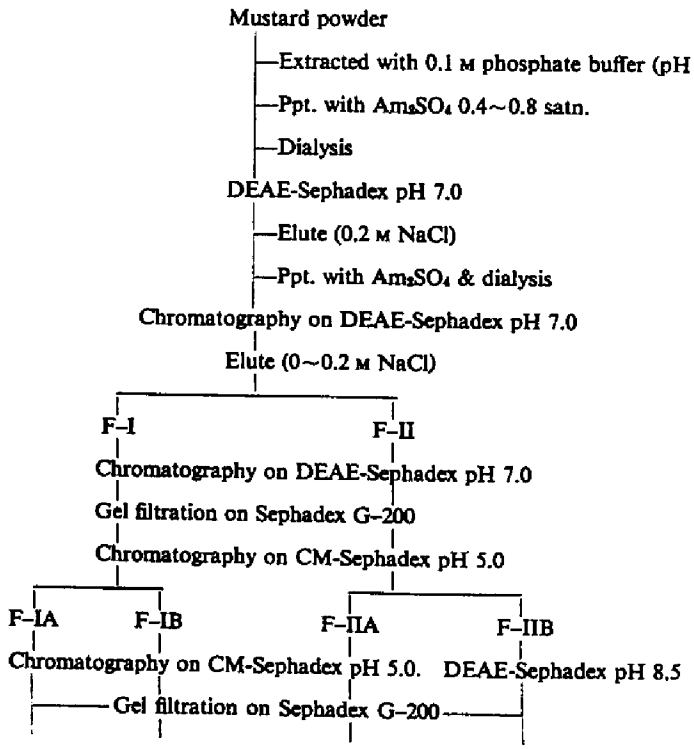


FIG. 1. Purification Steps of Plant Myrosinases.

Chromatography on Sephadex G-200. The molecular weights of the enzymes were further determined by the molecular sieve chromatography according to Andrewes.[12] Sephadex G-200 column (1.5×80 cm) was used with 0.01 M sodium phosphate buffer containing 0.1 M KCl, pH 7.0. Myoglobin, ovalbumin, serum albumin and γ-globulin were used as the standard proteins.

The elution volume (Ve) of each protein was estimated from an elution diagram, by extrapolating both sides of the protein peak to an apex. Determination of other gel-filtration parameters were according to Flodin and Porath,[13] Siegel and Monty,[14] Ackers,[15] and Andrews.[12]

Chemical analysis. Amino acid analysis was performed with a Yanagimoto LC-5S amino acid analyser. Duplicate samples were hydrolyzed for 22 and 72 hr under N_2 gas phase with glass distilled HCl at 110°C. Half-cystine was determined as cysteic acid after performic acid oxidation by the method of Moore.[16] Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton.[17]

Hexose content was estimated by the Tillman reaction[18] with mannose as the standard.

The molecular weight was determinated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn.[8]

Unless otherwise stated, all the purification procedures were conducted at 5°C.

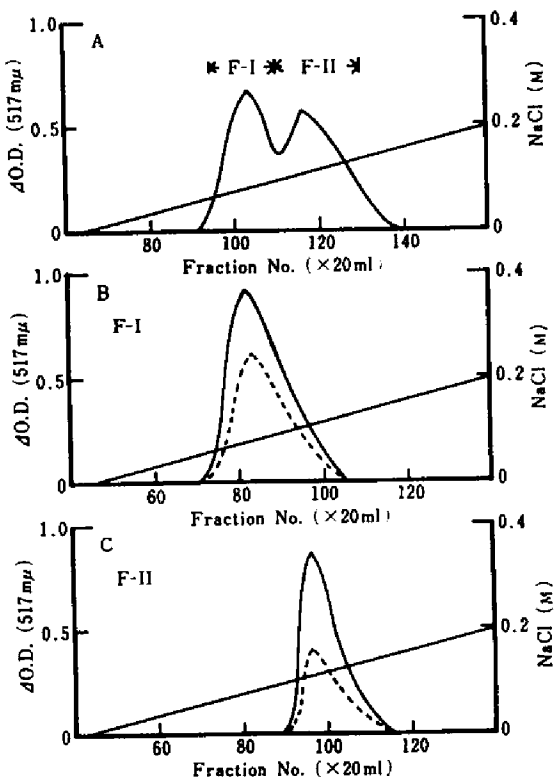


FIG. 2. A; Chromatography on DEAE-Sephadex B, C; Rechromatography on DEAE-Sephadex. (——) Myrosinase activity, (——) Protein.

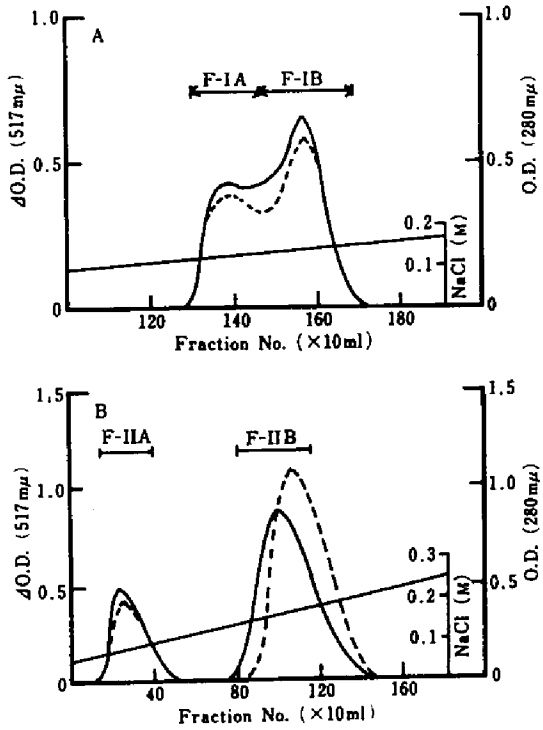


FIG. 3. Chromatography on CM-Sephadex. A; F-I Fraction, B; F-II Fraction. (——) Myrosinase activity, (– – –) Protein.

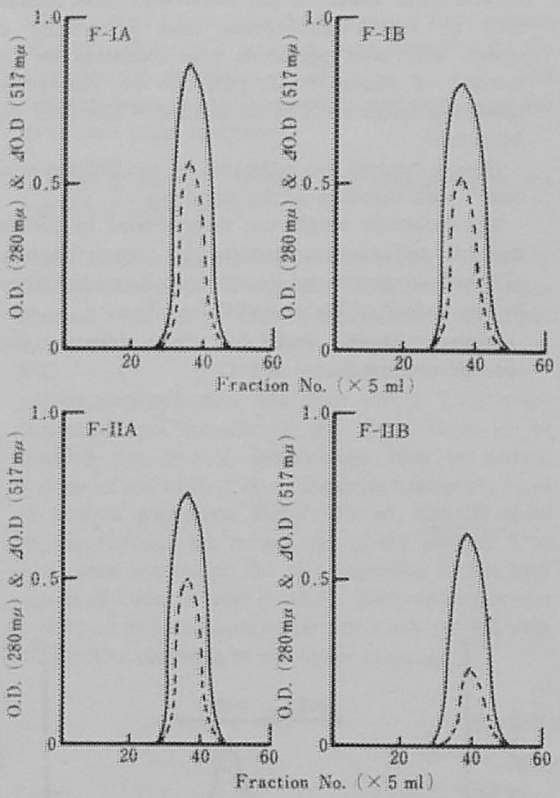


FIG. 4. Gel Filtration on Sephadex G-200.
(------) Myrosinase activity, (——) Protein.

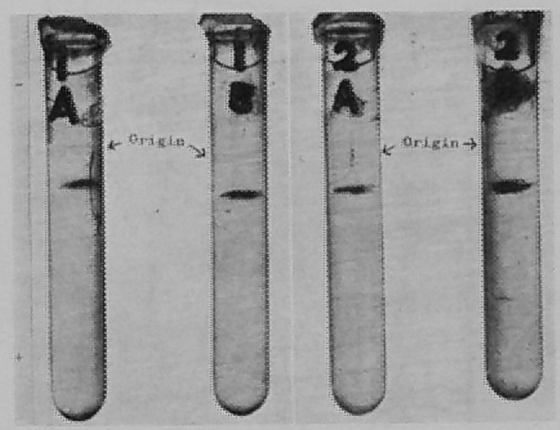


FIG. 5. Polyacrylamide Disc Gel-electrophoresis of Plant Myrosinases.

RESULT AND DISCUSSION

Homogeneity of the enzymes

All of the enzyme preparations (F–IA, B, F–IIA & F–IIB) were found to be pure as determined by gel-filtration, Fig. 4. Homogeneity of the preparations were also tested by discelectrophoresis and sedimentation experiments. As shown in Fig. 5, a single band was observed in the electrophoresis of the respective sample. The sedimentation patterns shown in Fig. 6 also confirmed the homogeneity of the preparations.

As shown in Fig. 7 the relationship between elution volume, *Ve*, and logarithm of molecular weight of the proteins is linear within the molecular weight range of $1.10^4 \sim 5.10^5$. A plot of the partition coefficient K_D versus logarithm of molecular weights also shows a straight line. The molecular weights obtained by these method were 150,000 for F–IA, F–IB & F–IIA and 125,000 for F–IIB (*Ve*/log mol. wt.), 155,000 for F–IA, F–IB & F–IIA and 125,000 for F–IIB (K_D/log mol. wt.).

Values for stokes radius, a, were calculated from a plot of the partition coefficient K_D versus the molecular radius (Fig. 8). The molecular radius', a(Å), for F–IA, B and F–IIA, and F–IIB were calculated to be 47 and 43, respectively.

The diffusion coefficient, D, of a protein was calculated from the familiar Stokes-Einstein diffusion relation:

$$D = \frac{kT}{6\pi\mu a} = \frac{RT}{6\pi\mu aN}$$

where a is the Stokes molecular radius, T, the absolute temperature, μ, the system viscosity, and K, the Boltzman constant which is equivalent to R/N, where R is the gas constant per mole, and N is Avogadro's number. The diffusion coefficients, D, (cm^2sec^{-1}) of F–IA, F–IB and F–IIA, and F–IIB were calculated to be 4.28×10^7 and 4.67×10^7 respectively.

The frictional ratio (f/fo) of a protein was

determined from the equation;

$$f/fo = a/(3\bar{v}M/4\pi N)^{1/3}$$

where $\bar{v}$ is the partial specific volume, which is assumed to be 0.741 after Svedberg and Peterson,[19] M the molecular weight obtained by gel filtration. The frictional ratios (f/fo) for F-IA, B, F-IIA, and F-IIB were calculated to be 1.33 and 1.29, respectively. From these results, it may be considered that F-IA, B & F-IIA shows remarkable resembrance to each other and that F-IIB has different properties.

Ultracentrifugal analysis

The molecular weight of the enzymes were estimated from the values obtained in three different concentrations. The values for F-IA, B & F-IIA were calculated to be in a range of 146,000 to 156,000. Therefore, average of molecular weight was determined to be 152,000. F-IIB was calculated to be 124,000.

High speed sedimentation velocity runs (see Fig. 6) at pH 5.0 and pH 7.0 showed symmetrical peaks. F-IA, B & F-IIA were calculated to be about 6.8 S and F-IIB, 5.8 S, respectively. Sedimentation coefficients do not seem to be dependent on pH. These data support the results of molecular weight determination by gel-filtration.

Isoelectric point

The results of isoelectric focusing are shown in Fig. 9. The peaks for activity and protein were found in the same positions. The isoelectric points of F-IA, B & F-IIA were about 4.6 and that of F-IIB was about 4.8.

Chemical properties of the enzymes

Carbohydrate contents. Myrosinases contain some hexose which could not be removed by either ion-exchange chromatography or dialysis. The hexose contents for F-IA, B, F-IIA & F-IIB, determined by the Tillman reaction,[18] were 15.8%, 17.8%, 22.5% and 8.6%, respectively, expressed as the mannose equivalent.

Amino acid composition. Table I summarizes the amino acid content of the enzymes. The amino acid composition of the enzymes were found to be alike on the whole, and that of F-IA, B & F-IIA were strikingly similar. F-IIB showed a comparative difference to others. It was found that the contents of aspartic acid and histidine were lower and that of glutamic acid, arginine and methionine were higher than those of others. This difference may be related to the fractionation dependent on ion-exchangeability, *i.e.* two peaks appeared on DEAE-Sephadex (see Fig. 2).

SDS-polyacrylamide gel electrophoresis

As can be seen from Fig. 10, the relationship between protein mobility and log molecular weight of the protein used in SDS-polyacrylamide gel system is linear within the molecular range of 1.10^4–6.10^4. It should be noted that applying the samples individually or collectively did not affect the mobility profile. The molecular weight F-IA, F-IB & F-IIA, and F-IIB were calculated to be about 40,000 and 30,000, respectively. This suggests that these myrosinases consisted of at least four subunits.

From the results obtained for myrosinase isozymes from mustard powder (Table II) by the methods, gel filtrations, ultracentrifugal analysis, electrophoretical analysis and amino acid analysis, it may be confirmed that F-IA, F-IB & F-IIA have striking resemblances and only F-IIB is rather different. Lower specific activity of F-IIB may be considered to be resulting from the difference of the composition of amino acid, lower content of carbohydrate and the change of protein structure with respect to molecular weight.

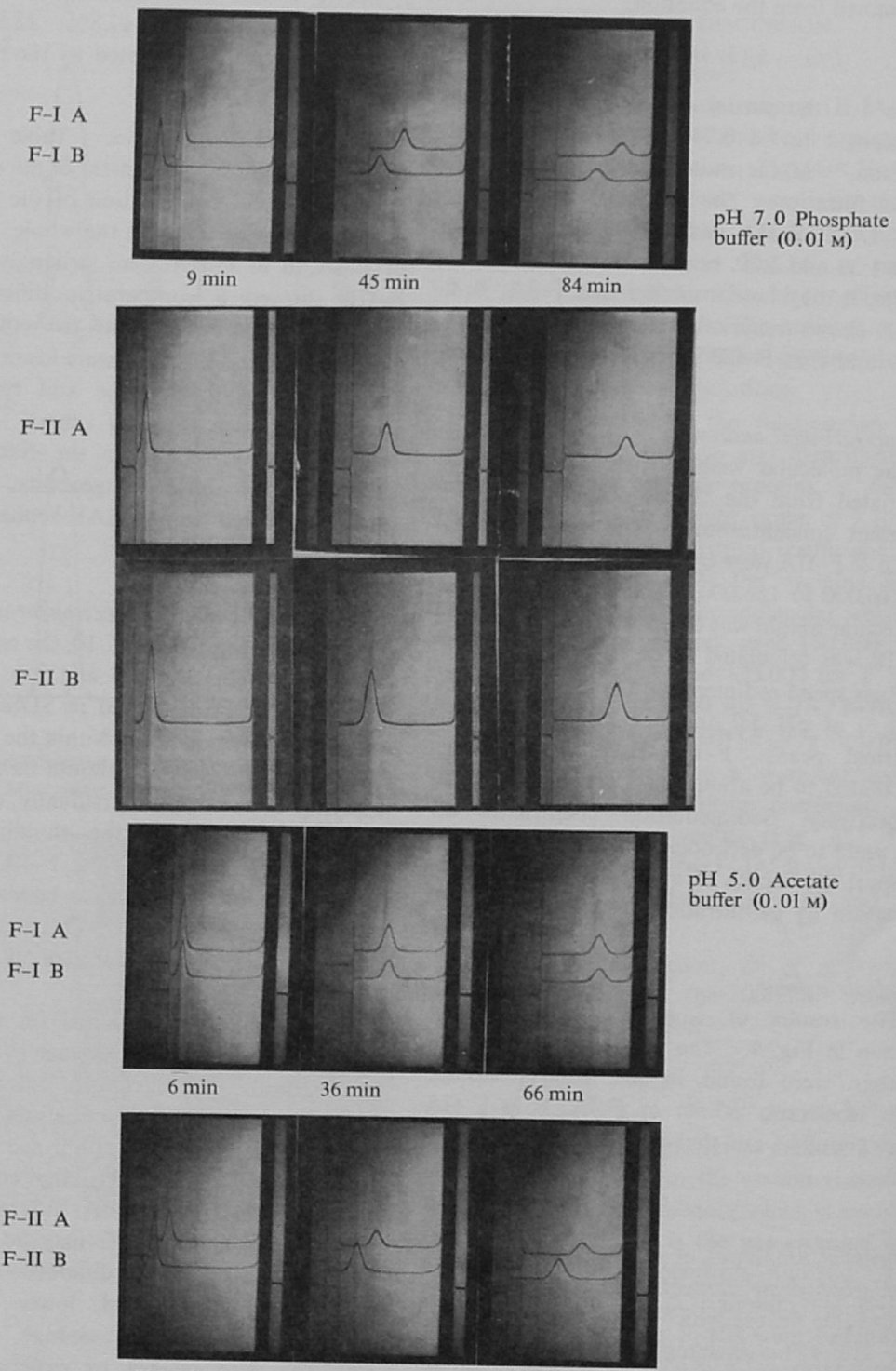


FIG. 6. Sedimentation Patterns of Plant Myrosinases.

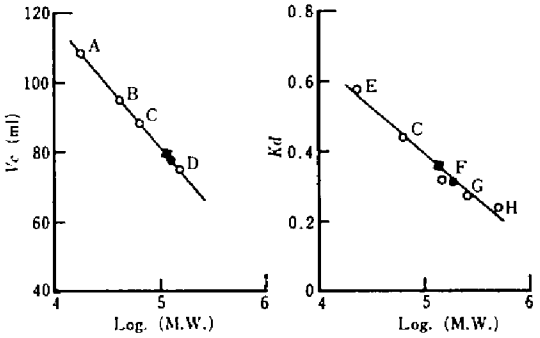


FIG. 7. Determination of Molecular Weight of Plant Myrosinases by Gel Filtration on Sephadex G-200.

A; Myoglobin, B; Ovalbumin, C; Serum albumin, D; Globulin, E; Hemoglobin, F; Alcohol dehydrogenase, G; Catalase, H; Urease.
(●) F-IA, B & F-IIA, (◆) F-IIB.

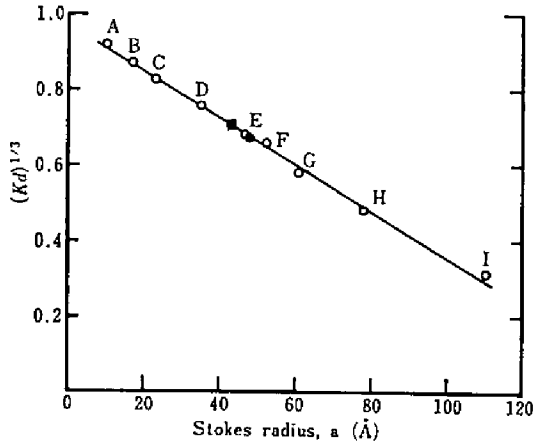


FIG. 8. Plot of Partition Coefficient *Kd* versus Molecular Radius.

A; Cytochrome c, B; Myoglobin, C; Hemoglobin, D; Serum albumin, E; Alcohol dehydrogenase, F; Catalase, G; Urease, H; Ferritin, I; Fibrinogen.
(●) F-IA, B & F-IIA, (◆) F-IIB.

These facts may have a relationship to the enzymatic activity. However, the rate of activation by ascorbate being the same as in others, it may be considered that the above facts do

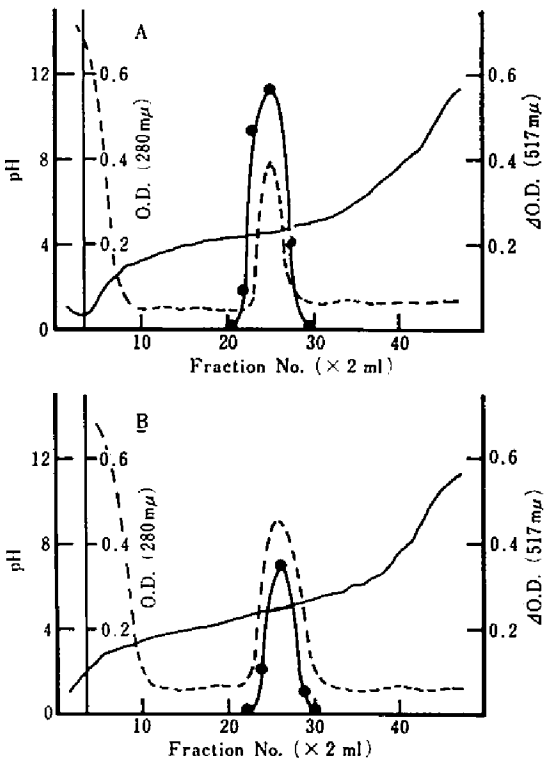


FIG. 9. Isoelectric Focusing of Plant Myrosinases.
A; F-IA, B & F-IIA, B; F-IIB.
(---) Protein, (●) Myrosinase activity, (—) pH

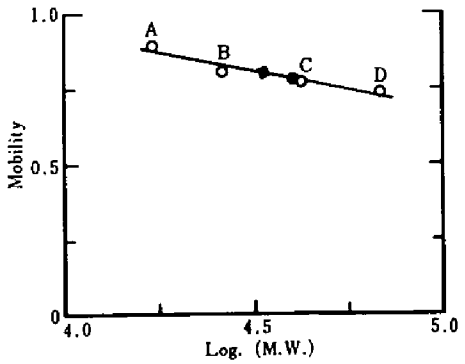


FIG. 10. Determination of Molecular Weight of Plant Myrosinases by SDS-Electrophoresis.

A; Myoglobin, B; Chymotrypsinogen, C; Aldolase, D; Serum albumin.
(●) F-IA, B & F-IIA, (◆) F-IIB.

TABLE I. AMINO ACID COMPOSITION OF PLANT MYROSINASES

Amino acid	F-IA	F-IB	F-IIA	F-IIB
		g amino acids/100 g protein		
Lys	6.07	5.88	6.24	5.41
His	2.75	2.64	2.31	1.82
Arg	4.86	4.53	4.50	7.49
Asp	14.50	14.24	14.83	8.12
Thr	5.15	5.2	5.24	4.76
Ser	5.6	5.36	4 97	6.0
Glu	8.49	8 09	8.87	11.96
Pro	5.13	5.3	5.76	7.04
Gly	4.63	4.4	4.4	5.8
Ala	3.09	3.21	3.17	4.46
Half-cys	1.78	1.52	2.33	1.35
Val	3.6	3.52	4.28	4.69
Met	1.55	1.42	1.94	3.58
Ileu	4.75	4.85	4.99	4.72
Leu	6.51	6.43	7.4	7.22
Tyr	6.43	6.49	2.72	2.56
Phe	4.63	4.37	4.72	4.40
Try[a]	3.61	6.5	4.74	1.85
Glucosamine	5.51	4.66	6.26	3.67
Hexose (as mannose) (%)	15.8	17.8	22.5	8.6

[a] Determined spectrophotometrically by the method of Goodwin and Morton.[17]

TABLE II. PHYSICOCHEMICAL PROPERTIES OF PLANT MYROSINASES

	F-IA	F-IB	F-IIA	F-IIB
$s_{20,w}$ pH 7.0 (S)	6.8	6.9	6.8	5.8
pH 5.0 (S)	6.7	6.8	6.8	5.9
Molecular weight (Sephadex G-200)				
Ve/log mole wt. (g)		150,000		125,000
Kd/log mole wt. (g)		155,000		125,000
Sedimentation equilibrium (g)		152,000		124,000
Stokes radius (Å)		47		43
Diffusion coeficient ($cm^2\ sec^{-1}$)		4.28×10^{7}		4.67×10^{7}
f/fo		1.33		1.29
Carbohydrate content (%)	15.8	17.8	22.5	8.6
Isoelectric point		4.6		4.8
Sp. activity		1000		100
+ASA/−ASA[a]		50——————100		

[b] ASA is L-ascorbic acid.

not affect the effected sites by ascorbate.

F-IA, B & F-IIA are quite alike but the rate of activation by ascorbate are not the same. It may be considered that the imperceptible differences of amino acid compositions and carbohydrate contents of the enzymes affect the rate of activation of enzymes by ascorbate.

Chapter 2

Functional Groups

Plant myrosinase is an enzyme which is specifically activated by ascorbate.[25,20,21] However, the functional groups of the enzyme are little known and there are only a few papers[2,5] describing that myrosinase was inhibited by *p*-mercuribenzoate.

In the previous Chapter,[22] I purified the enzyme and clarified the physical and chemical properties of myrosinase isoenzymes.

In this Chapter, I investigated the functional group of the purified myrosinase by the chemical modification, using reagents discriminating the states of amino acids in protein and discussed the activation mechanism of the enzyme by ascorbate.

MATERIALS AND METHODS

Chemical reagents. Monochlorotrifluoro-*p*-benzoquinone (CFQ) was purchased from Seikagaku Kogyo Co., Ltd. and other chemicals were obtained from Nakarai Chemicals Ltd. All reagents were of analytical grade, and used without further purification.

Substrate. Sinigrin, obtained from Nakarai Chemicals Ltd., was used as the substrate for myrosinase.

Enzyme preparation. The myrosinase solutoin from mustard powder was prepared by the methods described in the preceding Chapter, and F-IA fraction which was homogeneous chromatographically, Disc electrophoretically and ultracentrifugally, was used as the enzyme in this experiment.

Enzyme assay. The assay mixture contained 2.5 μ moles of substrate and enzyme solution in a total volume of 1 ml. Enzymatic activities were measured by the liberation of glucose, The reactions were carried out under conditions regarded to be zero order reactions.

Assay of protein. Protein was determined by the method of Lowry *et al.*[7]

Inhibition experiments. Unless otherwise stated, the incubation mixtures (500 μl) contained myrosinase (50~300 μg), inhibitors (1 mM) and buffer. After incubation for 20 min, aliquots (10~50 μl) were removed at specific times and the activities assayed.

Chemical modifications. The state of amino groups of myrosinase was determined by CFQ.[23] CFQ is stable in dioxane but is hydrolyzed gradually in water. Solutions (2~20 μl) of CFQ (100 mM) in dioxane were added to enzyme solutions (300 μl, 260 μg protein) and 0.2 M phosphate buffer, pH 8.0, in a total volume of 2 ml. The mixture thus obtained were incubated at 25°C for 15 min. During this incubation, the hydrolysis of CFQ and the reaction of CFQ with the enzyme proceeded simultaneously. A blank mixture was prepared in the same manner without enzyme solution, and the differences in absorbance at a wavelength (353 mμ) between the sample mixtures and the blank mixtures were measured with a Shimazu spectrophotometer model QV-50, using 1.0 cm cells. The moles of amino groups which reacted with CFQ in myrosinase were calculated, using $\Delta\varepsilon$ value (21,600 M^{-1} cm^{-1}).

The state of -SH groups of myrosinase was determined by Ellman reagent.[24] Solution (350 μl) of Ellman reagent (10^{-2} M) were added to enzyme solution (500 μl, 1000 μg protein) and 0.2 M phosphate buffer, pH 8.0, in a total volume of 3.3 ml. The mixtures thus obtained were incubated at 37°C. During this incubation, the hydrolysis of the reagent and the reaction of the reagent with the enzyme proceeded in parallel. A blank mixture was prepared in the same manner without enzyme solution, and the differences in absorbance at a wavelength (412 mμ) between the

sample and blank mixtures were measured at specified times. The moles of -SH groups which reacted with Ellman reagent in myrosinase were calculated, using $\Delta\varepsilon$ value (13,600 M^{-1} cm^{-1}). In the experiment where SDS was used, 0.2 M phosphate buffer containing 0.1% SDS was added.

Photooxidation of myrosinase. The enzyme (50~300 μg) was dissolved in 1 ml of 0.1 M phosphate buffer, pH 8.0, containing 0.01% methylene blue (MB). The reaction mixture was placed at distance of 40 cm from the front lens of a 750 W projector, and illuminated at 37°C for 15 min.

Analytical method. Amino acid analysis of photooxidized enzyme was carried out as follows: The lyophilized sample of photooxidized enzyme in 6 N HCl was sealed in evacuated tubes and hydrolyzed at 110°C for 24 hr. After the HCl had been removed by means of evaporation, the hydrolysate was applied to Amberite IR-120 column (1 × 5 cm), in order to remove the dye (methylene blue), and was eluted with 2 N ammonium hydroxide and eluate was evaporated to expel ammonia. The preparation was subjected to amino acid analysis in a Hitachi Automatic Amino Acid Analyzer, Type KLA 5.

RESULTS

Effect of various reagents on plant myrosinase

The functional group of plant myrosinase was investigated using reagents for discriminating the states of amino acids in protein. Effects of various reagents were summarized in Table I. FDNB and TNBS, which should generally react with the functional groups of protein such as fructose 1, 6-diphosphatase[25] and glutamate dehydrogenase,[26,27] inhibited myrosinase activity. CFQ, which reacts with amino groups, PCMB and Ellman reagent, which are well-known to be -SH reagents, and *p*-diazobenzene-sulfonic acid and dye-sensitized photooxidation, which are generally used to detect histidyl residue, inhibited the enzymatic activity strongly. Other reagents, N-acetylimidazol and iodine (tyrosine), DFP (serine), Koshland reagent and NBS (tryptophane), glyoxal and cyclohexanedione (arginine) and water soluble carbodiimide (carboxyl group), were all of no effect.

From the above results, amino, -SH and imidazol groups may be considered to be the functional groups of myrosinase.

Inhibitory effect of FDNB and TNBS on myrosinase

Inactivation of myrosinase by FDNB and TNBS were shown in Figs. 1 and 2. Inactivation rate by these reagents were at most 20~30% within 60 min, and they were increased by addition of ascorbate (10^{-3} M). Inactivation by FDNB did not occur when sinigrin (300 mM) and ascorbate (10^{-3} M) were added in the reaction mixture (Fig. 1, curve C).

CFQ incorporation during enzymic inactivation

CFQ is a new reagent, assigned[23] for discriminating various states of amino groups in protein. Figure 3 showed the relationship of residual activity and moles of reacted amino group versus mole between enzyme to CFQ concentration. Enzymatic activity was lost by the addition of CFQ (10^{-3} M) at 25°C for 15 min and five amino groups were modified.

Inhibitory effect of PCMB on myrosinase

Inactivation of myrosinase by PCMB was shown in Fig. 4. Myrosinase was inhibited by PCMB (10^{-5} M) at 37°C for 60 min but the rate of this inhibition was almost the same each other with or without ASA (10^{-3} M), which was different from that of FDNB and TNBS.

Ellman reagent incorporation during enzymic inactivation

Myrosinase was also inhibited by Ellman reagent,[24] which is well-known -SH reagent together with PCMB. Figure 5 showed the relationship of residual activity and moles of reacted sulfhydryl group versus mole of enzyme to incubation time with Ellman reagent. Enzymatic activity was inactivated by the addition of Ellman reagent (10^{-3} M) at 37°C for 30 min and then two sulfhydryl

TABLE I. EFFECTS OF VARIOUS REAGENTS AND METHOD

Enzymatic activity was measured by the liberation of glucose.

	Conc. (M)	Time (min)	Temp. (°C)	Remaining act. (%)
None				100
Fluorodinitrobenzene (FDNB)	10^{-3}	20	20	50
Trinitrobenzenesulfonic Acid (TNBS)	10^{-3}	20	20	90
Monochlorotrifluoro-*p*-benzoquinone (CFQ)	10^{-3}	20	25	10
p-Mercuribenzoate (PCMB)	10^{-4}	20	37	0
5-5′-Dithio-bis-(2-nitrobenzoic acid) (Ellman reagent)	10^{-3}	20	37	0
p-Diazobenzenesulfonic Acid	10^{-3}	30	25	15
Photooxidation		15	37	0
N-Acetylimidazole	10^{-3}	20	37	100
Diisopropylfluorophosphate (DFP)	10^{-3}	20	37	95
2-Hydroxy-5-nitrobenzyl bromide (Koshland reagent)	10^{-3}	20	25	99
N-Bromosuccinimide (NBS)	10^{-3}	20	37	95
Glyoxal	10^{-3}	20	25	100
Cyclohexanedione	10^{-3}	20	25	100
Water soluble carbodiimide	10^{-3}	20	37	100

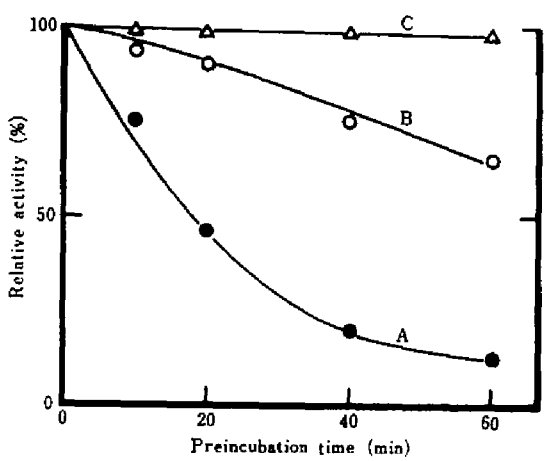


FIG. 1. Inactivation of Plant Myrosinase by FDNB.

100 μl (300 μg) of myrosinase were pre-incubated at 20°C with 1 mM FDNB in 0.2 M sodium bicarbonate buffer, pH 9.0, 10 mM (1 mM*) ASA and 300 mM sinigrin (both omitted in the sample of curve B and only sinigrin omitted in the sample of curve A*). The final volume was 0.5 ml. Aliquots (10 μl) were removed at different times and assayed.

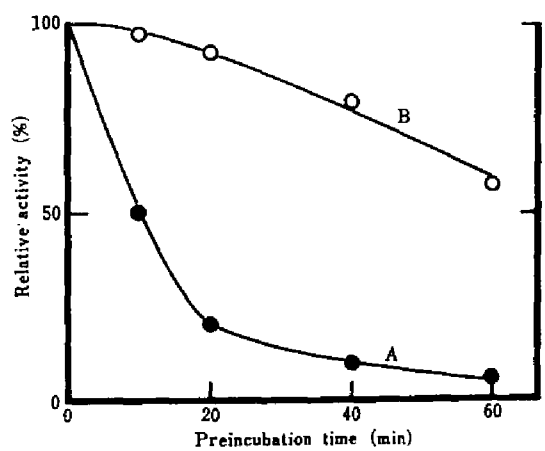


FIG. 2. Inactivation of Plant Myrosinase by TNBS.

100 μl (200 μg) of myrosinase were incubated at 20°C with 1 mM TNBS in 0.2 M sodium bicarbonate buffer, pH 9.0, and 1 mM ASA (omitted in the sample of curve B), in a total volume of 0.5 ml. Aliquots (20 μl) were removed at different times and assayed.

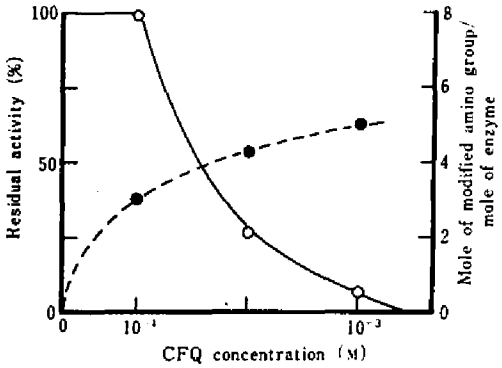


FIG. 3. CFQ Incorporation during Enzymic Inactivation.

300 μl (260 μg) of myrosinase were incubated at 25°C with 0.1 ~ 1 mM CFQ in 0.2 M sodium phosphate buffer, pH 8.0, for 15 min, in a total volume of 2.0 ml. Aliquots (20 μl) were removed and assayed.

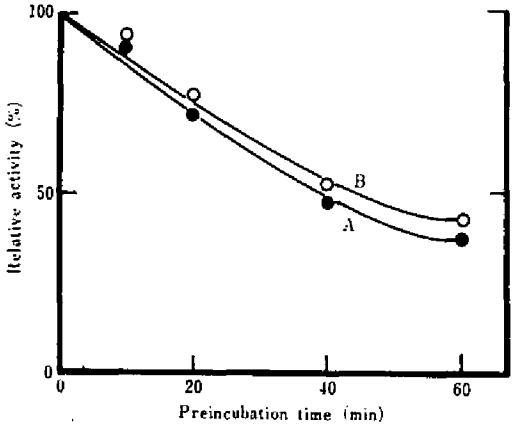


FIG. 4. Inactivation of Plant Myrosinase by PCMB.

100 μl (200 μg) of myrosinase were pre-incubated at 37°C with 1 mM PCMB in 0.2 M sodium phosphate buffer, pH 7.0, and 1 mM ASA (omitted in the sample curve B), in a total volume of 0.5 ml. Aliquots (20 μl) were removed at different times and assayed.

groups were modified. While four sulfhydryl groups were modified when the enzyme was denatured by SDS (0.1%).

Inhibitory effect of p-diazobenzenesulfonic acid on myrosinase

Inactivation of myrosinase by *p*-diazobenzenesulfonic acid was shown in Fig. 6. Myrosinase was inhibited by this reagent (10^{-3} M) at 25°C for 30 min, but enzymatic activity was protected in addition of sinigrin (300 mM). Inhibition rate by this reagent was very strong, but this reagent is well-known as a modifying reagent for histidyl- and tyrosyl residue.[28] As myrosinase was not affected by N-acetylimidazole[29] which react with tyrosyl residue, the inactivation by *p*-diazobenzenesulfonic acid may be considered to be dependent on the modification of histidyl residue. In order to confirm the above presumption photooxidation[17] of myrosinase, which is one of the modification of histidyl residue, was done.

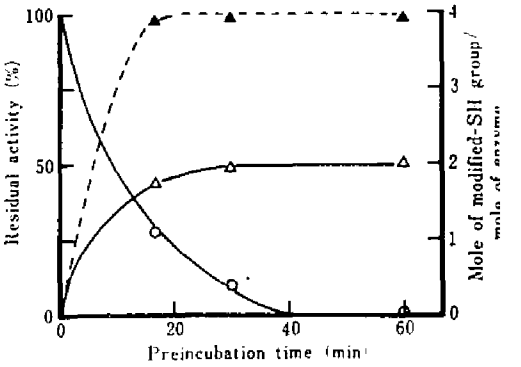


FIG. 5. Ellman Reagent Incorporation during Enzyme Inactivation.

500 μl (1 mg) of myrosinase were pre-incubated at 37°C with 1 mM Ellman reagent in 0.2 M sodium phosphate buffer, pH 8.0, and in the case of denaturation with SDS 0.2 M phosphate buffer containing 0.1% SDS was used. The final volume was 3.3 ml. Aliquots (10 μl) were removed at different times and assayed. (○) Enzymatic activity, (△) –SH modified (▲) –SH modified in the denatured enzyme by SDS

Dye-sensitized photooxidation of myrosinase

Plant myrosinase was photooxidized in phosphate buffer, pH 8.0 at 37°C in the presence of methylene blue (Fig. 7). This inactivation was protected in the presence of

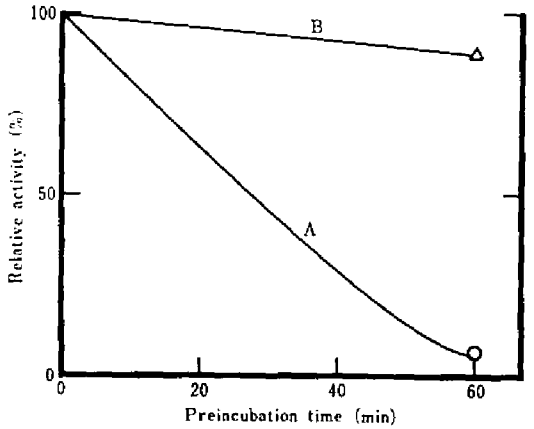


FIG. 6. Inactivation of Plant Myrosinase by *p*-Diazobenzenesulfonic Acid.

50 μl (100 μg) of myrosinase were incubated at 25°C with 1 mM *p*-diazobenzenesulfonic acid in 0.2 M sodium phosphate buffer, pH 8.0, for 30 min, and 300 mM sinigrin (omitted in the sample of curve A), in a total volume of 0.5 ml. Aliquots (20 μl) were removed and assayed.

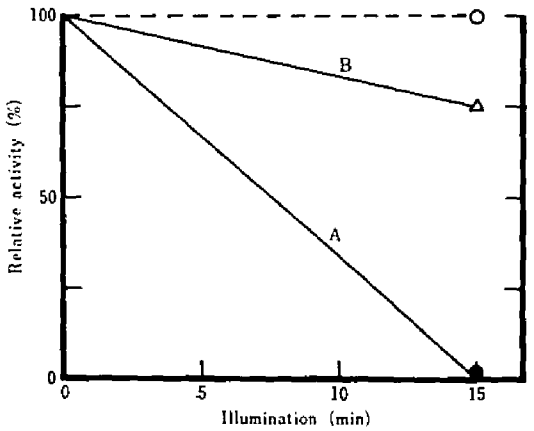


FIG. 7. Inactivation of Plant Myrosinase by Photooxidation.

100 μl (50~300 μg) of myrosinase were illuminated at 37°C with 0.01% methylene blue in 0.2 M sodium phosphate buffer, pH 8.0, and 300 mM sinigrin (omitted in the sample of curve A), in a total volume of 0.5 ml. Aliquots (50 μl) were removed and assayed. (○) Non-photooxidized enzyme

sinigrin (300 mM), and the enzyme was not photooxidized below 25°C.

In the dark, the enzyme was quite stable in the presence of methylene blue, and in light without the dye.

In general, it is known that histidine, methionine, tyrosine, tryptophane and cysteine are sensitive to photooxidation, but myrosinase activity was not affected by NBS and Koshland reagent. Therefore, amino acid composition of the photooxidized-enzyme was analyzed. Figure 8 showed the result of

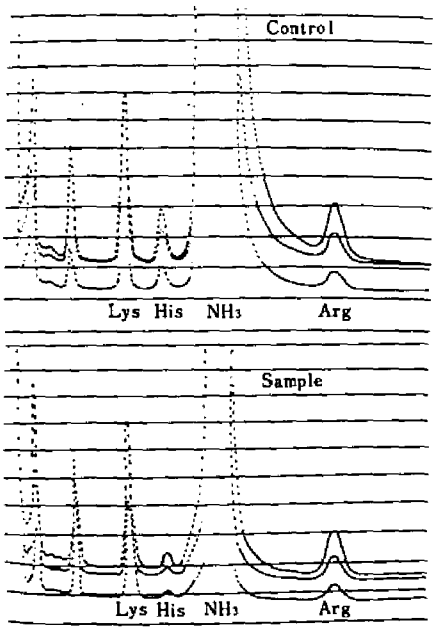


FIG. 8. Comparison of Amino Acid Composition of the Photooxidized and Non-treated Myrosinase.

150 μg of myrosinase were photooxidized at 37°C in 0.1 M sodium phosphate buffer, pH 8.0, containing 0.01% methylene blue for 30 min. Control was not photooxidized. Amino acid analysis was as described in the text.

amino acid analysis of the control and the photooxidized enzyme. The control was under the same conditions, but without illumination. The concentrations of the used enzymes were nearly the same. There were no differences between the control and the photooxidized enzymes, but only histidine content of the latter was found to be lower.

DISCUSSION

The functional groups of plant myrosinase were investigated using reagents which discriminate the states of amino acids in protein and by photooxidation. It was found that at least the amino groups, sulfhydryl groups and histidyl residues constitute the functional groups of plant myrosinase. FDNB and TNBS are well-known as the reagents which are used to determine α- and ε-amino groups in protein, but they are also used to study the functional group of proteins. Plant myrosinase is specifically activated by l-ascorbate (10^{-3} M)[5] but its activation mechanism is still indistinct. Tsuruo et al. had studied on the activation mechanism of plant myrosinase[21] by ascorbate, and confirmed that its activation was not based upon the oxidation and reduction by ascorbate, but was based on the changes of conformation of enzyme in addition of ascorbate. The results obtained by FDNB and TNBS support the above conclusion, that is, groups sensitive to FDNB and TNBS may be considered not to be very reactive in the native state of the enzyme but to become more reactive in the presence of ascorbate. The reactive groups to FDNB and TNBS may be considered to be amino groups.

Myrosinase was inactivated when five amino groups were modified by CFQ, but its activity still remained when three amino groups were modified. Therefore, it may be considered that two remaining amino groups are necessary to the activity of myrosinase. The different rate of inhibition by FDNB, TNBS and CFQ may be considered to be dependent on the shape and dimension of these reagents.

The inactivation by –SH reagents were strong. Plant myrosinase have at least four sulfhydryl groups of which two sulfhydryl groups concern with the activity of myrosinase. As the rate of inhibition by PCMB was nearly the same each other with or without ascorbate, sulfhydryl groups which take part in the enzymatic activity may be thought to be re active in a native state in the presence o absence of ascorbate.

Histidyl residue is also one of the functiona groups of myrosinase. However, owing t the interaction of the reagent used wit ascorbate, in the method employed, the effec of ascorbate on the state of histidyl residue i protein could not be investigated.

From the above results, amino groups, sulf hydryl groups and histidyl residue may b considered to be in the active center of plan myrosinase, and thus the modification of an individual group or residue by the specific reagents caused inactivation. It may be considered that the above groups and residue are located closely to each other in the three dimensional structure of the protein. Activation by ascorbate may be assigned to the conformational changes of myrosinase caused by ascorbate: Even a slight conformational change of myrosinase caused by ascorbate may cause the surface exposition of the amino groups, or the dissociation and association of protein, because plant myrosinase consists of at least four subunits.[22]

Chapter 3

Approach to the Interaction of L-Ascorbic Acid to the Enzyme

Nagashima *et al.*[5] found that the plant myrosinase was activated strongly by l-ascorbic acid (ASA). Schwimmer[20] and Ettlinger *et al.*[21] investigated this phenomenon. The latter concluded that ASA behaved as a reversibly dissociable base, closely connected with the nucleophilic group of thioglucosidase. Kojima *et al.*[31] studied the changes of the reaction product of the enzymatic hydrolysis of mustard oil glucoside in the presence of ASA at low pH.

Tsuruo et al. described that the oxidation-reduction reaction of ASA had nothing to do with the activation reaction of myrosinase.[2] The presence of the effector site for ASA was presumed by the kinetic measurements and the instabilization of the enzyme by ASA on heating. I also reported[32] that the inhibitions of plant myrosinase by fluorodinitrobenzene (FDNB) and trinitrobenzenesulfonic acid (TNBS) were stronger in the presence of ASA.

From the above results, the author speculated that the activation mechanism of plant myrosinase by ASA may be considered to be the result of slight conformational change of myrosinase or the dissociation and association of the enzyme.

Some experiments were carried out to elucidate the activation mechanism of this enzyme. Enzyme reaction and assay of enzymatic activity were described previously.[1] Myrosinase (F-IA fraction)[22] was used as the enzyme source.

The activation was observed above 5×10^{-5} M ASA concentration, and the maximum activation was around 10^{-3} M ASA (Fig. 1). Activation gradually decreased at higher concentrations. The decrease of activation at higher concentration can be explained by assuming that ASA acts as a competitive inhibitor of plant myrosinase.[2]

In the previous Chapter, I described that the plant myrosinase consisted of at least four subunits. Therefore, there is a possibility that the activation of myrosinase depend on the dissociation-association mechanism of the enzyme protein by the addition of ASA. I investigated the presence of dissociation and association of the enzyme by gel-filtration and ultracentrifugal analysis.

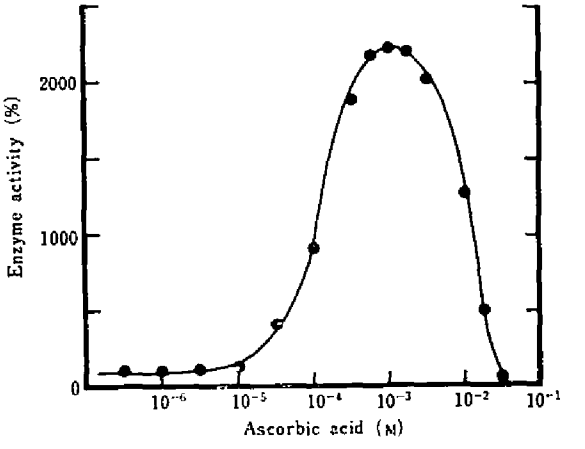


FIG. 1. Effect of Ascorbic Acid.

Enzyme reactions were carried out in the system containing 2.5 μmoles of the substrate (sinigrin) in 1.0 ml of water at pH 7.0 and 37°C. The myrosinase activity was measured as the liberation of sulfate by titration with a recording pH-stat, Radiometer Model SBR2/SBU1/TTT1 Autotitrator.[6]

Figure 2 shows the gel-filtration chromatogram of myrosinase on Sephadex G-150 with and without ASA (10^{-3} M). The enzyme was eluted at the same position with or without ASA.

Sedimentation studies were carried out in a Spinco model E ultracentrifuge.

High speed sedimentation velocity runs with or without ASA (10^{-3} M) showed symmetrical peaks with the same value of 6.8 S. (Fig. 3).

These results indicated that the enzyme was neither dissociated nor associated by ASA. That is, the activation of myrosinase by ASA did not result in the dissociation and association of the enzyme by addition of ASA.

In the previous Chapter, I investigated the functional group of myrosinase by using reagents for discrimination the state of amino acid and found that the amino, sulfhydryl groups and the histidyl residue were associated with the active site of myrosinase.[33]

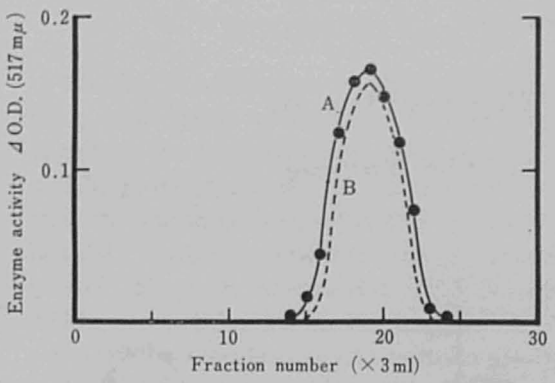


FIG. 2. Gel-filtration on Sephadex G-150 with and without Ascorbic Acid.

Gel-filtration on Sephadex G-150 column (1×90 cm), equilibrated with 0.01 M sodium phosphate buffer containing 0.01 M KCl, pH 7.0, and 1 mM ASA (omitted in the sample of curve A), was performed at 5°C. One ml of the enzyme dissolved in the same buffer (ASA omitted in the sample of curve A) was subjected to the column and eluted with the respective buffers. Enzymatic activities were measured by the liberation of glucose,[6] as described previously.[7]

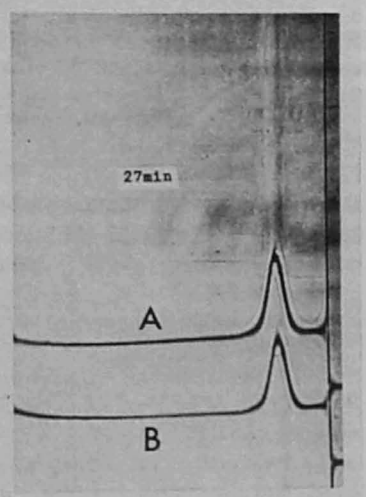


FIG. 3. Sedimentation Patterns of Plant Myrosinase with and without Ascorbic Acid.

Sample solutions contained 0.01 M sodium phosphate buffer, pH 7.0, 0.1 M KCl, 1 mM ASA (omitted in the sample of peak A), and 6.5 mg of myrosinase. Measurements of the sedimentation coefficient were performed at a speed of 59,780 rpm in a double sector cells at 20°C.

The myrosinase was also found to be inactivated strongly in the presence of ASA than without it by TNBS.

The state of amino groups of myrosinase was determined by TNBS. Solution (1 ml) of TNBS (3.3×10^{-3} M) were added to enzyme solution (200 μl, 688 μg protein) containing 4% sodium bicarbonate buffer, pH 9.0, 0.5 ml of ASA (6×10^{-3} M), in a total volume of 3.0 ml. The mixture thus obtained were incubated at 20°C for 30 min and the reaction was stopped by the addition of 1 ml of SDS (10%) and HCl (1 N). During this incubation time, the hydrolysis of TNBS and the reactions of TNBS with the sample of enzyme proceeded in parallel. A blank mixture was prepared in the same manner without enzyme solution, and the differences in absorbance at a wavelength (340 mμ) between the sample and the blank mixture were measured. The moles of amino groups which reacted with TNBS in myrosinase were calculated, using $\Delta\varepsilon$ value which is 10,000 $M^{-1}cm^{-1}$. As indicated in Table I, myrosinase was not inactivated by TNBS (10^{-3} M) for 30 min without ASA, but the enzyme was rapidly inhibited by TNBS within 30 min in the presence of ASA (10^{-3} M). And fifteen amino groups were modified in the absence of ASA, but only thirteen amino groups showed modification in the presence of ASA. This indicates that the functional groups become more reactive due to the conformational change of the protein resulted by ASA, and would be modified by TNBS.

TABLE I. NUMBER OF AMINO GROUPS PER PROTEIN MOLECULE REACTING WITH TNBS IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID

Myrosinase was incubated with the reagent for 30 min and aliquots (10 μl) were assayed for activity. Enzymatic activities were measured by the liberation of glucose, as described previously.[7]

Incubation mixture	Incubation time (min)	Amino groups reacted	Enzyme activity (%)
1 mM TNBS	30	14.9	90
1 mM TNBS + 1 mM ASA	30	13.2	45

These results supported the conclusion that the activation mechanism of myrosinase by ASA depend on the slight conformational change of the protein resulted by ASA, and is not due to the dissociation and association mechanism of myrosinase.

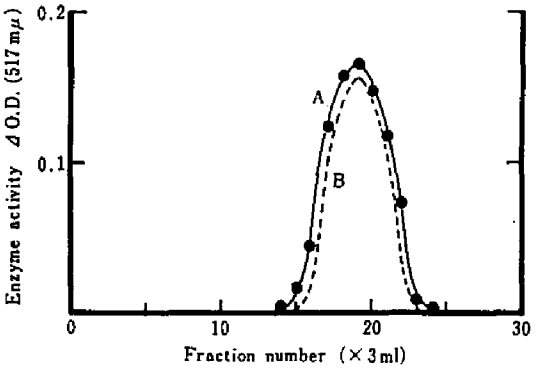


FIG. 2. Gel-filtration on Sephadex G-150 with and without Ascorbic Acid.

Gel-filtration on Sephadex G-150 column (1×90 cm), equilibrated with 0.01 M sodium phosphate buffer containing 0.01 M KCl, pH 7.0, and 1 mM ASA (omitted in the sample of curve A), was performed at 5°C. One ml of the enzyme dissolved in the same buffer (ASA omitted in the sample of curve A) was subjected to the column and eluted with the respective buffers. Enzymatic activities were measured by the liberation of glucose,[6] as described previously.[7]

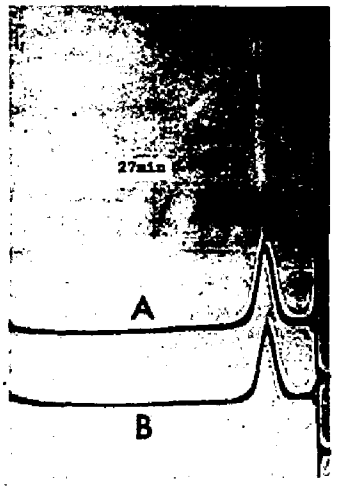


FIG. 3. Sedimentation Patterns of Plant Myrosinase with and without Ascorbic Acid.

Sample solutions contained 0.01 M sodium phosphate buffer, pH 7.0, 0.1 M KCl, 1 mM ASA (omitted in the sample of peak A), and 6.5 mg of myrosinase. Measurements of the sedimentation coefficient were performed at a speed of 59,780 rpm in a double sector cells at 20°C.

The myrosinase was also found to be inactivated strongly in the presence of ASA than without it by TNBS.

The state of amino groups of myrosinase was determined by TNBS. Solution (1 ml) of TNBS (3.3×10^{-3} M) were added to enzyme solution (200 μl, 688 μg protein) containing 4% sodium bicarbonate buffer, pH 9.0, 0.5 ml of ASA (6×10^{-3} M), in a total volume of 3.0 ml. The mixture thus obtained were incubated at 20°C for 30 min and the reaction was stopped by the addition of 1 ml of SDS (10%) and HCl (1 N). During this incubation time, the hydrolysis of TNBS and the reactions of TNBS with the sample of enzyme proceeded in parallel. A blank mixture was prepared in the same manner without enzyme solution, and the differences in absorbance at a wavelength (340 mμ) between the sample and the blank mixture were measured. The moles of amino groups which reacted with TNBS in myrosinase were calculated, using $\Delta\varepsilon$ value which is 10,000 $M^{-1}cm^{-1}$. As indicated in Table I, myrosinase was not inactivated by TNBS (10^{-3} M) for 30 min without ASA, but the enzyme was rapidly inhibited by TNBS within 30 min in the presence of ASA (10^{-3} M). And fifteen amino groups were modified in the absence of ASA, but only thirteen amino groups showed modification in the presence of ASA. This indicates that the functional groups become more reactive due to the conformational change of the protein resulted by ASA, and would be modified by TNBS.

TABLE I. NUMBER OF AMINO GROUPS PER PROTEIN MOLECULE REACTING WITH TNBS IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID

Myrosinase was incubated with the reagent for 30 min and aliquots (10 μl) were assayed for activity. Enzymatic activities were measured by the liberation of glucose, as described previously.[7]

Incubation mixture	Incubation time (min)	Amino groups reacted	Enzyme activity (%)
1 mM TNBS	30	14.9	90
1 mM TNBS + 1 mM ASA	30	13.2	45

These results supported the conclusion that the activation mechanism of myrosinase by ASA depend on the slight conformational change of the protein resulted by ASA, and is not due to the dissociation and association mechanism of myrosinase.

Chapter 4

Binding of Ascorbate to the Enzyme and the Interaction of Ascorbate with the Functional Groups

In the preceding chapters,[22, 32] I purified the enzyme and clarified the physical and chemical properties of myrosinase isoenzymes, the myrosinases from white mustard (Sinapis alba L.) and rapeseed (Brassica napus L.) have been isolated and characterized by Janson et al.,[33, 34] who showed the existence of a number of isoenzymes, also found by Henderson and McEwen,[35] and studied the functional groups of plant myrosinase using reagents which discriminate the states of amino acids in protein and found that amino group, –SH group and histidyl residue constitute the active sites of the enzyme. I also found that the activation mechanism of myrosinase by ascorbate depend on the slight conformational change of the protein resulted by ASA, and is not due to the dissociation and association mechanism of myrosinase. I reported that β-glucosidase activity was not activated by ASA but was inhibited competitively at higher concentration.

In this chapter, I determined the binding of ASA ligands to myrosinase by the dialysis rate technique and studied spectrophotometrically the interaction of ASA with the enzyme by the inhibitory effect by the chemical modification, using reagents discriminating the states of amino acids, on myrosinase and β-glucosidase activities in connection with the activation of the enzyme and discussed the relationship between the active center and the activation mechanism of the enzyme by ascorbate.

MATERIALS AND METHODS

Chemical reagents L-Ascorbic-1-C^{14} acid was purchased from New England Nuclear Corporation and 2-methoxy-5-nitrotropone (MNT) was purchased from Sankyo Co., Ltd. Other chemicals were obtained from Nakarai Chemicals Ltd. All reagent were of analytical grade, and used without further purification.

Substrate Sinigrin, obtained from Nutritional Biochemicals Corporation, was used as the substrate for myrosinase and p-nitrophenyl β-D-glucoside (Sigma) as the substrate for β-glucosidase.

Enzyme preparation The myrosinase solution from yellow mustard powder was prepared by the methods described in the previous Chapter[22], and F-IA fraction was used as the enzyme in this experiment.

Enzyme assay The assay mixture contained 2.5 μ moles of substrate and enzyme solution in a total volume of 1 ml. Enzymatic activities were measured by the liberation of glucose, sulfate and p-nitrophenol.[1, 6] The reactions were carried out under conditions regarded to be zero order reactions.

Assay of protein Protein was determined by the method of Lowry et al.[7]

Inhibition experiments Unless otherwise staled, the incubation mixtures (500 μl) contained myrosinase (50~300 μg), inhibitors PCMB (10^{-4}M), DEP (10^{-2}M) and MNT (10^{-3}M) and buffer.
Aliquots (5~20 μl) were removed at specific times and the activities assayed.

Chemical modifications The state of amino groups of myrosinase was determined by MNT.[36] Solution (5 μl) of MNT (100 mM) in N,N'-dimethylformamide was added to enzyme solution (200 μl, 1.0 mg protein)

and 0.2 M phosphate buffer (pH 8.5) in a total volume of 1.5 ml. The mixture thus obtained was incubated at 25°C for 60 min. The product was passed through a column of Sephadex G-50 suspended in water to remove excess reagent and by-products. Myrosinase appeared as a yellow colored fraction. The moles of amino groups which reacted with MNT in myrosinase were calculated, using $\Delta\epsilon$ value ($2.07 \times 10^4 M^{-1} cm^{-1}$) at 420 mμ.

The modification of tryptophane residues was determined by HNBB.[37] Solution (5 μl) of HNBB (100 mM) in acetone was added to enzyme solution (100 μl, 500μg protein) and 0.5 M acetate buffer (pH 5.0) in a total volume of 205 μl. The reaction mixture was incubated at 35°C for 45 min. The product was done by the same method as MNT. The moles of tryptophane residues which reacted with HNBB in myrosinase were calculated, using $\Delta\epsilon$ value ($1.80 \times 10^4 M^{-1} cm^{-1}$) at 410 mμ (pH 10).

Photometrical measurements Measurements of absorption spectra and differential spectra were carried out with a Hitachi dual wave length spectrometer 356 B.

Rate dialysis method Rate dialysis was carried out by the method of Colowick and Womack,[38] using the apparatus (Fig. 1.) consist of a dialysis with an upper chamber, containing the enzyme and labeled substrate, separated by a membrane from a lower chamber, through which buffer is pumped at a constant rate and from which the effluent is sampled for measurement of radioactivity.

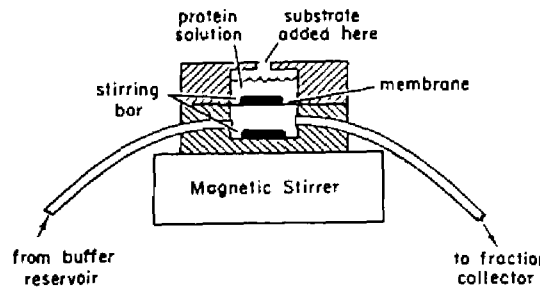


Fig. 1 Diagram of the Apparatus for Measuring Substrate Binding by Rate of Dialysis.

The dialysis cell was adapted from the Technilab cell (Bel-Art Products) for continuous flow dialysis (1 ml size). The discshaped upper chamber (19 mm in diameter x 5 mm) was altered in two ways. It was deepened to 9 mm to make the capacity about 2.5 ml and a hole (5 mm in diameter) was drilled to the outside to permit additions of small volumes of substrate or other agents to the enzyme solution (1.5 ml) during the course of a binding measurement. The lower chamber (19 mm in diameter x 10 mm) has a capacity of 2.8 ml and is completely filled with buffer solution which is pumped through at a constant rate of 20 ml per hrs. The membrane, a square cut from ordinary cellophane dialysis tubing (Visking Company), is clamped between the Lucite blocks, which are held together by stainless steel screws. The contents of both chambers are mixed by means of small magnetic stirring bars; the bar in the top chamber rests on the membrane. The inner diameter of the tubing leading from the lower chamber to the fraction collector is small, 0.015 inch, in order to minimize dead space. Aliquots (0.5 to 0.8 ml) from the fraction collector are diluted in scintillant (PPO 10 g, POPO 0.25 g naphthalene 100 g, dioxane 11) for liquid scintillation counting (Packard Tri-Carb 2002 Scintillation Spectrometer). The dialysis apparatus has been used satisfactorily either in the cold room (T=5°) or at room temperature (T=25°).

RESULTS

Measurement of ASA binding to myrosinase

Figure 2 illustrates the procedure used and the results obtained in a binding measurement. The first aliquot of ligand was labeled with ^{14}C, and subsequant additions contained nonradioactive ligand. This procedure[38, 39] ensures optimal counting rates over a wide range of ligand concentrations. Note that in the absence of diminution of free ligand concentration by binding, dilution, or loss by dialysis, the addition of nonradioactive ligand would not alter the ^{14}C dialysis rate, because the effect of increased ligand concentration is exactly cancelled by the corresponding decrease in specific activity. The two curves were obtained under identical conditions except that myrosinase was omitted from the curve labeled "without enzyme." It is apparent that at low ASA concentrations the dialysis rate of ASA is lowered considerably by the presence of myrosinase, indicating that a large proportion of the total ASA is protein-bound and therefore non-dialyzable.

To evaluate the binding constant and the number of binding sites, the data from Fig. 2 (Curve with enzyme) are treated as follows. The steady state concentration of isotope found in the effluent at any given substrate concentration is taken as a measure of the fraction of the total

substrate in the upper chamber in the freely diffusible state. When excess unlabeled substrate is added, the radioactivity in the effluent is taken to be that corresponding to 100% of the substrate in the free state. Thus, dividing any observed value by this maximum value gives the fraction of the substrate free at a given concentration. Values for the concentration of free (F) and bound (B) substrate then easily derived. For example, from Fig. 2, at a total ASA concentration of 2.21×10^{-3}M, the fraction of ASA free is 1100/1300 or 0.182, so that the values of F and B are 1.8×10^{-3} and 3.41×10^{-4}M, respectively. These values can be used to determine the dissociation constant, K_{diss}, by applying the equation

$$K_{diss} = \frac{(n - B) \cdot F}{B}$$

where n is the concentration of the total binding sites on the protein. It enzyme of known purity and n value is used, one measurement of F and B at a single substrate concentration can be used to determine K_{diss}. With enzyme of unknown purity or n value, a series of F and B values at different substrate concentrations serves to evaluate both K_{diss} and n. In this case, one makes a Scatchard-type plot of B versus B/F, and determines K_{diss} from the slope and n from the intercept, according to the linear form of the above equation

$$B = n - K \cdot B/F$$

Such a plot is shown in Fig. 3a. Myrosinase appears to have 4 sites per molecule which bind ASA rather strongly ($K_D = 0.1 \times 10^{-4}$M), and at least one additional site which binds ASA less strongly ($K_D = 0.9 \times 10^{-4}$M). Myrosinase activity is maximumly activated with the concentration of ASA (1 mM) and then the enzyme binds the four ASA molecules. (Fig. 3.B).

Effect of ASA analogues on myrosinase activity

As shown in Table I, the myrosinase activity was not activated by ASA analogues tested. Nagashima and Uchiyama[5] showed that myrosinase was not activated by reducing reagents

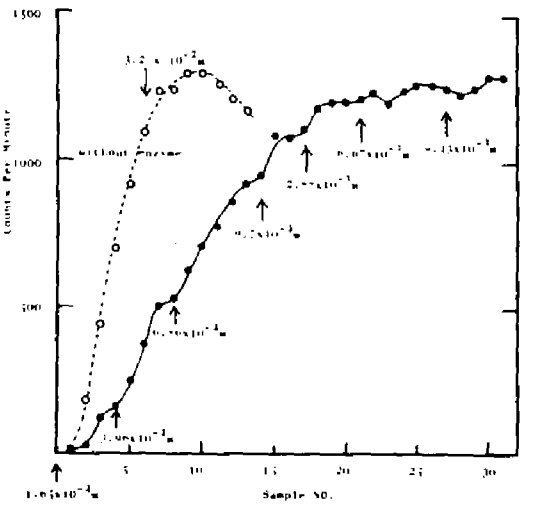


Fig. 2 Measurement of Ascorbic Acid at Varying ASA Concentrations.

The time course of two dialysis rate measurements, without and with enzyme, is shown, but only the latter is required in a routine measurement. The specific activity of the ^{14}C-ASA added at the beginning was 2.72 mCi per mmole. After radioactivity in the effluent reached a steady state, increments of unlabeled ASA were added to give the total concentrations indicated under the arrows. Protein concentration 11 mg per ml ($=0.73 \times 10^{-4}$ M). Buffer 0.05 M phosphate, pH 7; T = 20°C.

(glutathione, cysteine, BAL, Gallic acid) except ASA and Ettlinger et al.[21] reported that the enzyme was activated by 2-o-methyl-L-ascorbate which didn't keep the effect of reducing power. These results showed that ascorbate was not acting on the conventional reducing agent.

Differential spectra of myrosinase

Figure 4 shows the differential spectra of myrosinase with or without ASA. The enzyme protein is comfornationally changed by the addition of ASA.

Figure 5 shows the absorption and differential spectra of the chemical modified myrosinase by MNT. Approximately 1.5 amino residues were appeared on the surface of the enzyme by adding ASA (10^{-3}M).

The spectra of HNBB modified myrosinase

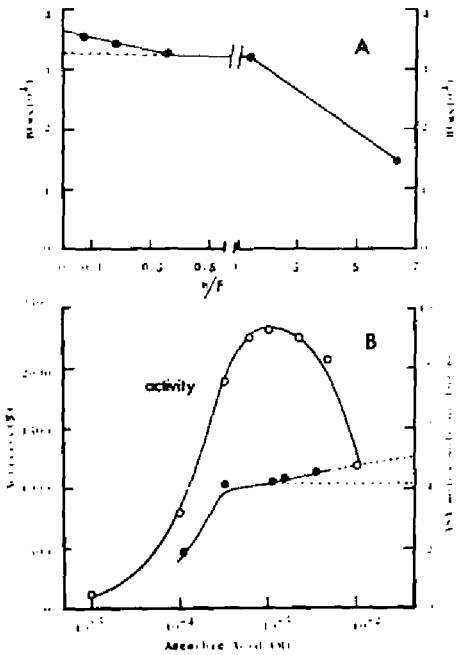


Fig. 3 Plot of the Data Derived from the Steady State Values in Fig. 2.

Showing Binding of ^{14}C-ASA to Plant Myrosinase (A) and Relationship of the Activation to the Binding of ASA (B).

were shown in Fig. 6. About 2.3 tryptophanyl residues were buried in the molecule when ASA was added (10^{-3}M).

These results showed the myrosinase was conformationally changed by the addition of ASA.

Inhibition by PCMB

As shown in Fig. 7, myrosinase (A, B) and β-glucosidase (C, D) activities were strongly inhibited by PCMB (10^{-4}M) at 25°C for 30 min. Inactivation rate of myrosinase activities were increased by the addition of ascorbate (10^{-3}M) when the activities were measured, but that of β-glucosidase activities were contrary. From these results, it is considered that sulfhydryl groups are located at the active center of myrosinase.

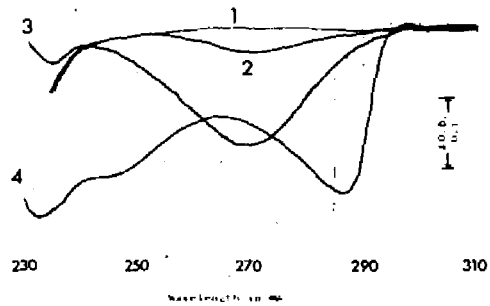


Fig. 4 Differentaial Spectrum of Myrosinase With and Without ASA.

Protein concentration is 1 mg/ml in 0.05 M phosphate buffer and 1 mM EDTA, pH 7.0, 20°C, in both compartments. Concentrations of ASA are:
1) None; 2) 5 x 10^{-5} M; 3) 1.0 x 10^{-4} M;
4) 1.0 x 10^{-3} M.

Inhibition by DEP

Inactivation of myrosinase by DEP was shown in Fig. 8. Inactivation rates of myrosinase activities by this reagent were stronger than β-glucosidase activities, and especially after the enzyme

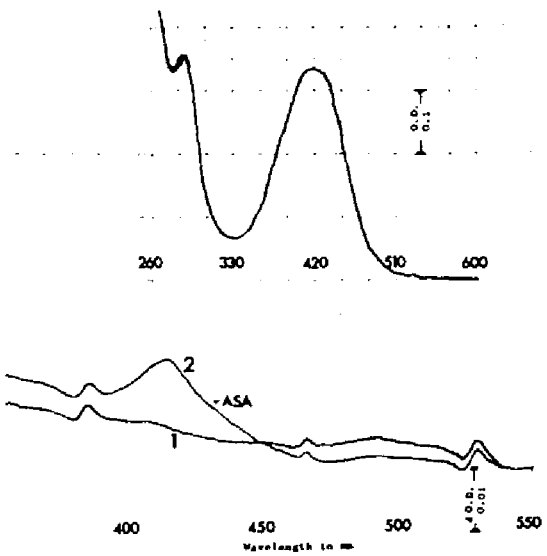


Fig. 5 Absorption and Differential Spectra of the Chemical Modified Myrosinase by MNT.

Protein concentration is 0.25 mg/ml in 0.05 M phosphate buffer, pH 8.5, 20°C. Concentrations of ASA are:
1) None; 2) 1.0 x 10^{-3} M.

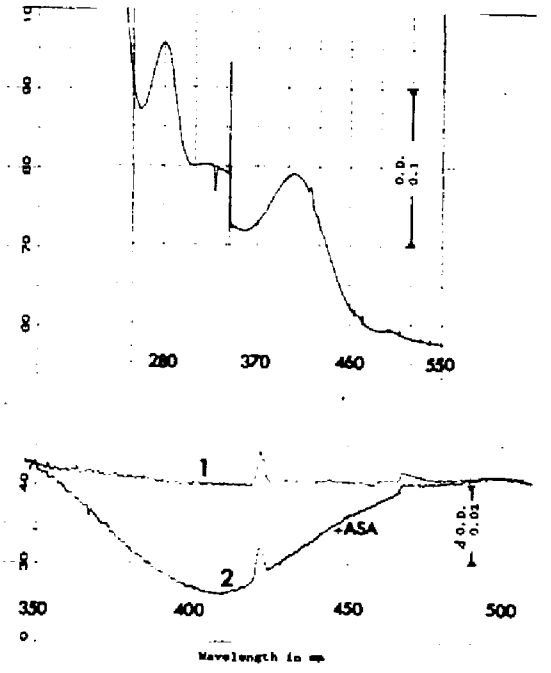
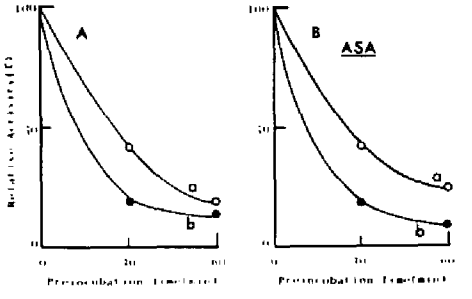


Fig. 6 Absorption and Differential Spectra of the Chemical Modified Myrosinase by HNBB.

Protein concentration is 0.15 mg/ml in 0.05 M carbonate buffer, pH 10, 20°C. Concentrations of ASA are: 1) None; 2) 1.0×10^{-3} M.



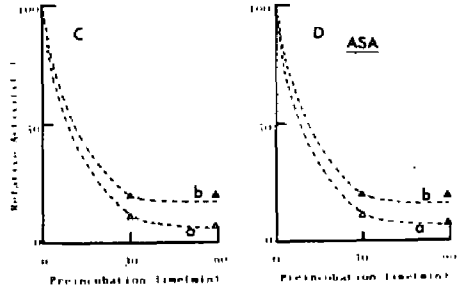
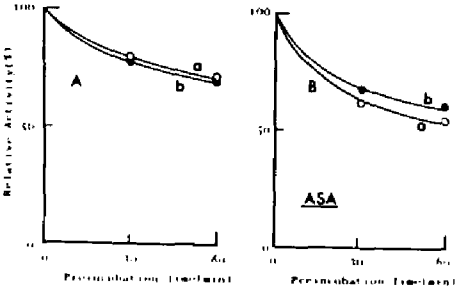


Fig. 7 Inactivation of Plant Myrosinase by PCMB.

100 μl (300 μg) of myrosinase were pre-incubated at 25°C with 0.1 mM PCMB in 0.2 M potassium phosphate buffer, pH 7.0, and 1 mM ASA (omitted on the sample of A and C), in a total volume of 0.5 ml. Aliquots (5~20 μl) were removed at different times and assayed. Enzymatic reactions were carried out in the system containing 2.5 μmoles of substrate, 0.2 mmole of phosphate buffer, 20 μl of the sample in Curves a (5 μl in Curves b), pH 7.0, and in Curves b 1 mM of ASA was added.



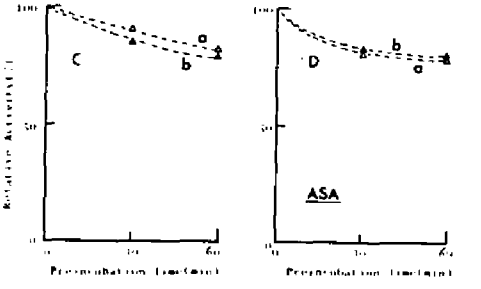


Fig. 8 Inactivation of Plant Myrosinase by DEP.

100 μl (300 μg) of myrosinase were pre-incubated at 25°C with 10 mM DEP in 0.2 M acetate buffer, pH 5.0, and 1 mM ASA (omitted in the sample of A and C), in a total volume of 0.5 ml. Enzymatic reactions were carried out as described in Fig. 7.

was preincubated at 25°C with 10 mM DEP and 1 mM ASA, myrosinase activity was strongestly inhibited when the enzymatic activity was measured without ASA. This showed that histidyl residues in the protein were affected by the addition of ASA.

Inhibition by MNT

Inactivation of myrosinase by MNT was

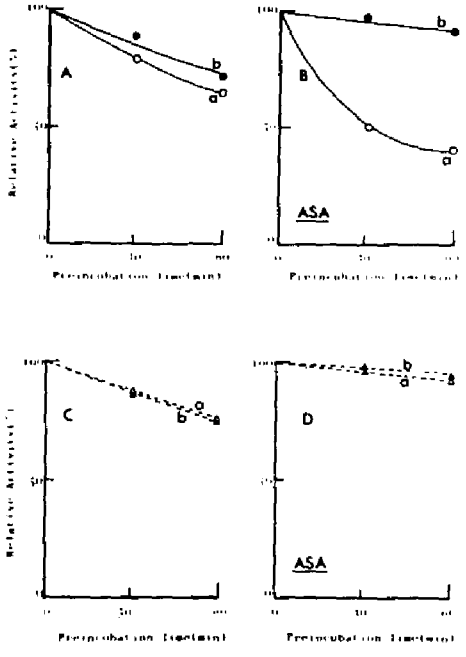


Fig. 9 Inactivation of Plant Myrosinase by MNT.

100 μl (300 μg) of myrosinase were pre-incubated at 25°C with 1 mM MNT in 0.2 M potassium phosphate buffer, pH 8.5, and 1 mM ASA (omitted in the sample of A and C), in a total volume of 0.5 ml. Enzymatic reactions were carried out as described in Fig. 7.

Table I Effect of the Analogues of Ascorbic Acid on Myrosinase Activity

Enzymatic activities were measured by the liberation of sulfate.

AsA analogue (10^{-3} M)	Relative activity (%)
None	100
L-ascorbate	2560
Dehydro-L-ascorbate	122
D-araboascorbate	140
Glucoascorbate	92
Ascorbyl-palmitate*	170
Ascorbyl-stearate*	85
Ascorbyl 2,6-dipalmitate*	47

* AsA analogues were dissolved in dimethylformamide (10^{-1} M).

shown in Fig. 9. Inactivation rates of myrosinase and β-glucosidase activities by this reagent were almost same, but in the case of B very interesting results was obtained. Differing from other reagents, myrosinase activity was strongly inhibited when the enzymatic activity was measured without ASA, but, the activity was not affected by this reagent when the activity was measured with ASA. This phenomenon was not occured

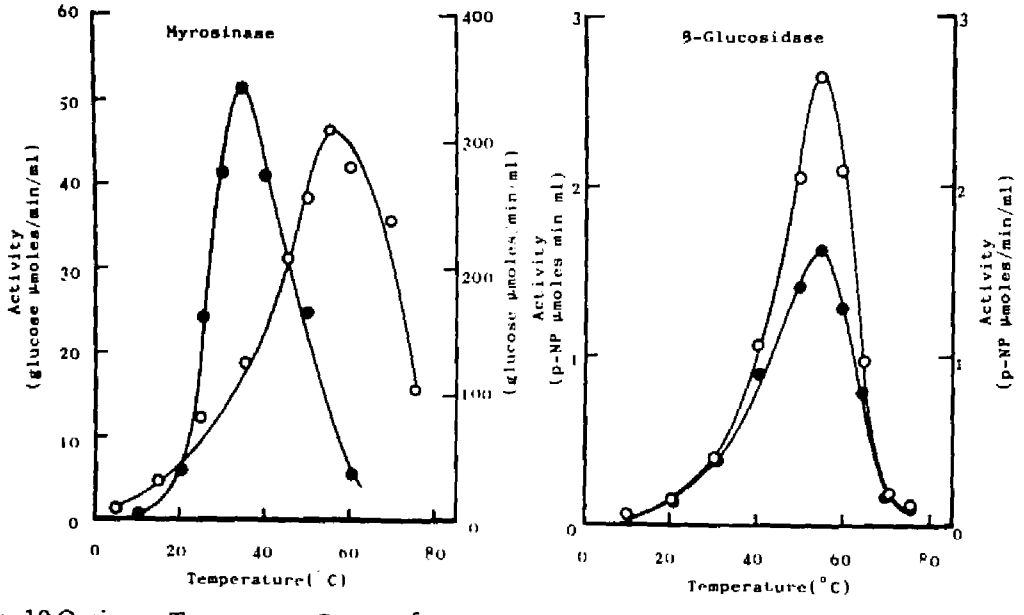


Fig. 10 Optimum-Temperature Curves of Myrosinase and β-glucosidase Activities of Plant Myrosinase.

—o—o— without ASA, —●—●— with ASA

Reactions were carried out at 7.0 for 15 min without ASA or for 6 min with ASA.

in β-glucosidase activity. This showed that amino residues in the protein were located at the region which was changed by ASA.

Optimum temperature of myrosinase

Optimum temperature of myrosinase with or without ASA were shown in Fig. 10. Optimum temperature of myrosinase activity was at about 55°C without ASA, but that with ASA was at about 35°C. This changing of optimum temperature suggested that the structure of myrosinase protein was change by the addition of ASA. Optimum temperatures of β-glucosidase activity were the same each other with or without ASA and they were at about 55°C which was same to that of myrosinase without ASA. This suggested β-glucosidase activity was not accelerated by ASA.

DISCUSSION

Tsuruo et al. assumed in the previous paper[2] two sites on the surface of the enzyme, namely the substrate site for mustard oil glucoside and the effector site for ascorbate. Furthermore, applying Pigman's model [40, 41] for β-glucosidase to the enzyme, two areas were distinguished[4] in the substrate site, one for adsorbing the glycon moiety of the substrate and the other for adsorbing its aglycon moiety. It was also pointed out that a possible structural alteration of the enzyme protein caused by the binding of ascorbate to the effector site exerted its effect only on the area for aglycon moiety. Since the binding of ascorbate to the effector site exerts no influence on p-NPG hydrolysis, the glycon moiety of p-NPG, which differs from mustard oil glucoside in aglycon moiety, is adsorbed only by the area for glycon moiety in the substrate site but not by the area for aglycon moiety. That is, p-NPG hydrolysis is neither accelerated nor its kinetics altered by the presence of ascorbate. Ettlinger et al.[22] described the hydrolysis of 2-4-dinitrophenyl β-thioglucoside and desulfoglucocapparin by mustard myrosinase is not accelerated by the presence of ascorbate. This observation can be similarly explained. As these thioglucosides have different aglycons from mustard oil glucoside, they can combine with the enzyme only at the area for glycon moiety in the substrate site, hence the hydrolyses of the two thioglucosides were not influenced by ascorbate.

Sinigrin competes with p-NPG at the same site of the enzyme protein. Consequently, p-NPG hydrolysis was competitively inhibited by sinigrin and the Ki values of p-NPG hydrolysis for sinigrin are nearly equal to the Km values for sinigrin hydrolysis.[1] Moreover, the former was altered by the absence or presence of 10^{-3}M ascorbate parallelly with the alteration of the latter. The effects of the presence of 10^{-3}M ascorbate on the kinetic constants of the enzyme are summarized in Table II. This table also shows the constancy of the Km value for p-NPG hydrolysis and the Ki values for salicin and glucose [4] in sinigrin hydrolysis. It is evident from the table that the binding of sinigrin to the enzyme was affected by the presence of ascorbate but that the binding of β-glucoside or glucose remained unaffected. These discussions concerning the substrate site and the effector site of the enzyme are represented as a schematic model in Fig. 11.

The experimental results obtained in the present chapter can be explained as follows (see Figs. 7, 8 & 9). Since both myrosinase and β-glucosidase activities were strongly inhibited by PCMB with or without ASA, sulfhydryl groups are considered to be essential to the catalytic action of myrosinase, but the inhibition rate of myrosinase activity by DEP and MNT were rather strongly inhibited than that of β-glucosidase activity, and then the enzyme which was treated by MNT with ASA was strongly inactivated when the enzymatic activity was measured without ASA, but, the enzyme was not inhibited when the activity was determined with ASA. Thus phenomenon didn't occure on the β-glucosidase

Table II Effect of Ascorbate of Kinetic Constants of Myrosinase

Ascorbate (M)	Hydrolysis of sinigrin			Hydrolysis of *p*-NPG	
	Km value for sinigrin (M)	*Ki* value for glucose (M)	*Ki* value for salicin (M)	*Km* value for *p*-NPG (M)	*Ki* value for sinigrin (M)
0	1.8×10^{-4}	1.5	1.8×10^{-1}	2×10^{-3}	2×10^{-4}
10^{-3}	9.3×10^{-4}	1.5	1.8×10^{-1}	2×10^{-3}	9×10^{-4}

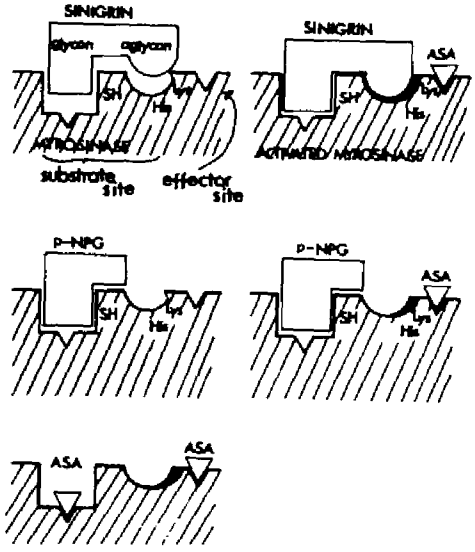


Fig.11. SCHEMATIC MODEL OF MYROSINASE ACTION

activity. From these facts, it is considered that at least amino and histidyl residues are situated on the region which is altered by the addition of ASA, that is, it is close to aglycon moiety and the locations of them are changed to accelarate the enzyme action by the binding of ASA to the effector site. On the other hand, as sulfhydryl reagent inhibited strongly to myrosinase and β-glycosidase activities, sulfhydryl groups are considered to be situated closely to glycon moiety. These also shows that p-NPG combines with the enzyme only at the area for glycon moiety in the substrate site, hence the hydrolysis of p–NPG was not influenced MNT and DFP. The above conclusion is also supported by which optimum temperature of myrosinase activities were changed by the addition of ASA but that of p-NPG hydrolysis were the same in spite of presence or absence of ASA.

That is, although the enzyme is capable of combining sinigrin at the substrate site, the fit of sinigrin to the site would be imperfect in the absence of ascorbate. This imperfect molecular fit of sinigrin would be improved by a possible alteration in the structure [3, 4] of the enzyme by ascorbate-binding at the effector site. And since the alteration of the enzyme's protein structure caused by the binding of ascorbate exerted no effect on the area which adsorbed glycon moiety in the substrate site, and since p-NPG, possessing a different aglycon from sinigrin, was combined by its glycon moiety to the glycon moiety-adsorbing area, the binding state of p-NPG would be that of an improved fit regardless of the absence or presence of ascorbate.

The inhibitory effect of ascorbate at high concentrations is accountable by the binding of ascorbate at a different site from the specific effector site and that of ascorbate was competitive. The Ki value (higher than the Km value for ascorbate in the activation of the enzyme [2]) indicates that ascorbate-binding occurs at a different site.

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PART II

Studies on the Fungous Myrosinase

Chapter 1

Production, Purification and Some Properties of the Extracellular Myrosinase from Aspergillus sydowi

Myrosinase hydrolyzes mustard oil glucoside to mustard oil (isothiocyanate), glucose and sulfate. This enzyme has been found in fungi and bacteria as well as in plants. Reese *et al.*[1] prepared the enzyme from the culture broth of *Aspergillus sydowi*, after a screening test of various fungi and bacteria, and described some of its characteristics. Kojima and Tamiya[2] also investigated the enzyme of the same organism. Oginsky *et al.*,[3] after testing enzymatic activity among intestinal bacteria, used *Paracorobactrum aerogenoides* as the active organism for their experiments.

These studies were, however, made on crude preparations and the reaction mechanism of microbial enzymes was not investigated.

As for the original nature of plant myrosinase, several authors[4~7] have concluded that the enzyme is not a mixture of thioglucosidase and sulfatase but a single β-thioglucosidase. However, the nature of microbial myrosinase still remained uncertain.

In this Chapter, I prepared a highly purified enzyme from *Aspergillus sydowi* and described its characteristics. The original nature of the fungous myrosinase is also discussed in comparison with the results of plant myrosinase.

MATERIALS AND METHODS

Strain. *Aspergillus sydowi* IFO 4284 was kindly supplied by the Institute for Fermentation, Osaka.

Cultural condition. The strain was precultured at 29°C on a 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 $MgSO_4 \cdot 7H_2O$), pH 6.5. After incubation for 3 days, mycellia were harvested and washed two times with water. Thirty grams of the wet mycellia were inoculated to 1 liter of the synthetic medium which consisted of 0.1% yeast extract, 0.2% potassium dihydrogen phosphate, 0.1% ammonium sulfate, 0.09% sodium nitrate, 0.09% magnesium sulfate, 0.9% glucose and 200 ml/liter of mustard extract. Cultivation of the organism was carried out in 2 liter flasks containing 600 ml of the medium for 2 weeks at 29°C under shaking on a reciprocal shaker.

Substrate. Sinigrin was prepared by the method of Nagashima and Uchiyama[8] from commercial yellow mustard (*Brassica juncea*) seed and used as the substrate of the fungous myrosinase.

Enzyme preparation. After cultivation the contents of flasks were combined and filtered. To each liter of the filtrate, 662 g of ammonium sulfate was slowly added with constant stirring. The resulting precipitate was collected by centrifugation and was dialyzed against 0.01 M phosphate buffer, pH 7.0.

Enzyme assay. Enzymatic activity was determined as thioglucosidase and sulfatase activities, respectively. Both reactions were carried out in the system containing 2.5 μmoles of the substrate and 0.1 μmole 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 measured by 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,[9] with a previously described modification.[7] The data obtained were corrected by multiplying the factor, 1.25, to eliminate the color development caused by the substrate itself.[7]

Sulfate was determined by titration with 0.01 N sodium hydroxide, using a recording pH-stat, Radiometer Model SBR2/SBU1/TTT1 Autotitrater at pH 7.0 under a nitrogen gas stream.

Assay of protein. Protein concentrations were determined by the method of Lowry *et al.*[10]

Inhibition experiments. Testing reagents and the enzyme were incubated at pH 7.0 and 37°C for 30 min before each experiment. Enzymatic reactions were initiated by an addition of substrate.

RESULTS

Effect of pH on growth

A typical culturing process of the fungus is shown in Fig. 1. The pH value of the culture broth fell below 4 at the early stage of the cultivation. When pH of the broth was not controlled, myrosinase activity rapidly decreased with the fall of pH. Therefore, the broth was adjusted to about pH 7 with 1 N sodium hydroxide. In this way enzyme production was accomplished smoothly.

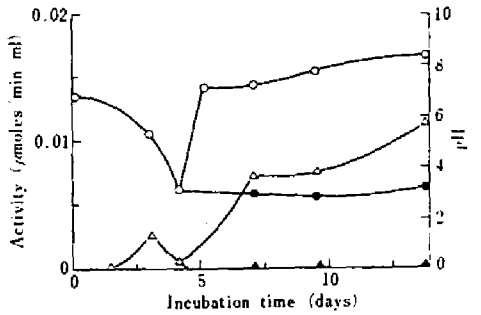


FIG. 1. Culturing Process of *Aspergillus sydowi*.

(○) pH of the broth was occasionally adjusted to neutrality with 1 N NaOH. (●) pH adjustment of the broth was not carried out. (△) Myrosinase production under pH adjustment and (▲) under no pH adjustment of the broth. The enzymatic activities were measured by the liberation of glucose.

Purification of the fungous myrosinase

1) Chromatography on DEAE-cellulose. The myrosinase solution (1100 ml, total protein 3.56 g), dialyzed against 0.01 M phosphate buffer, pH 7.0, was charged on a DEAE-cellulose (400 ml) column which was equilibrated with the same buffer. The adsorbed protein was eluted batchwise with 2 liters of the same buffer containing 0.1 M sodium chloride. The eluted protein was precipitated by 90% saturation with ammonium sulfate and was dialyzed against the same buffer. The dialyzate was applied to a DEAE-cellulose column (2×16 cm) equilibrated with the same buffer. Elution of the protein was performed with a linear gradient from 0 to 0.2 M sodium chloride in 0.01 M phosphate buffer (Fig. 2). The enzyme fractions indicated by the horizontal arrow in the figure were pooled, precipitated by 90% saturation with ammonium sulfate and then dialyzed against the same buffer.

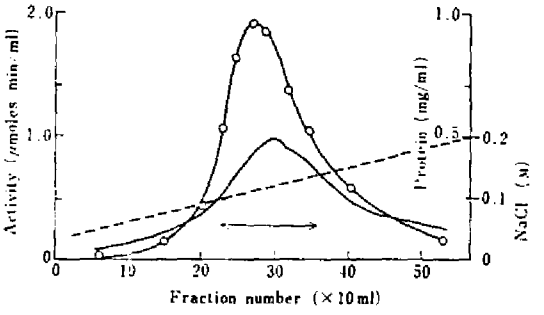


FIG. 2. Chromatogram of Fungous Myrosinase on a DEAE-Cellulose Column (2×16 cm).

(○) Enzymatic activity measured by glucose liberation, (—) Protein, (---) NaCl conc. Fractions indicated by the horizontal arrow were pooled. The experimental conditions are described in the text.

2) Chromatography on DEAE-Sephadex. The dialyzate (30 ml, total protein 42 mg) was applied on a DEAE-Sephadex column (1×15 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0. Elution of the protein was performed with a linear gradient from 0 to 0.2 M sodium chloride in the same buffer. The peaks of

enzymatic activity and protein were eluted at nearly the same location (Fig. 3). The enzyme fractions indicated by the horizontal arrow were pooled and dialyzed against the same buffer. This dialyzate was used as the purified preparation in the following experiments. Purification steps and yields of

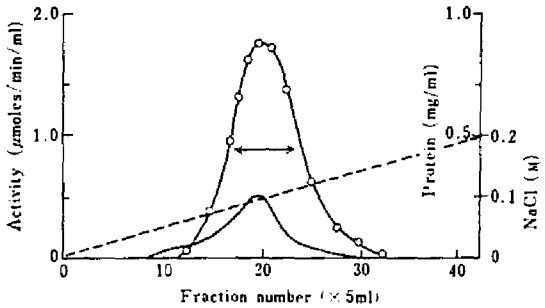


FIG. 3. Chromatogram of Fungous Myrosinase on a DEAE-Sephadex Column (1×15 cm).

(○) Enzymatic activity measured by glucose liberation, (—) Protein, (—) NaCl conc. Fractions indicated by the horizontal arrow were pooled. The experimental conditions are described in the text.

the fungous myrosinase are summarized in Table I.

The enzyme was purified approximately 150 fold from the culture broth and its yield was 22%.

General characteristics of the fungous myrosinase

Figures 4, 5 and 6 show pH-activity, pH-stability and temperature-stability curves, respectively. There are few differences in these curves between the thioglucosidase and sulfatase activities. Optimum pH of both activities was at about pH 7. They were stable in the pH range from 5.5 to 8.5 and at temperature below 45°C.

Figure 7 shows the Lineweaver-Burk plot of the fungous myrosinase. The *Km* value was calculated to be 3.6×10^{-3} M.

The chromatographic behavior of the fungous myrosinase

Since the two activities of the enzyme, thioglucosidase and sulfatase activities, showed no difference in pH-activity, pH-stability and temperature-stability curves, an attempt to separate the enzyme to thioglucosidase and

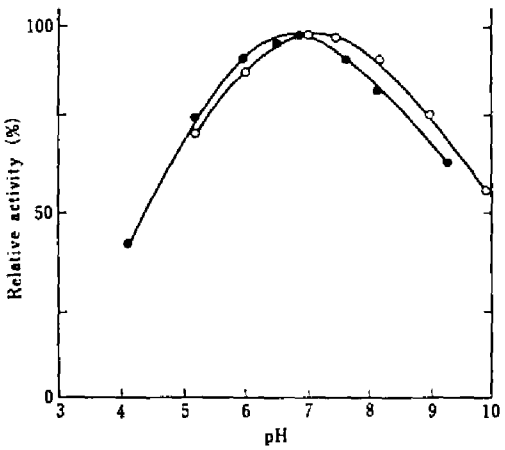


FIG. 4. pH-Activity Curves of Fungous Myrosinase.

(○) Thioglucosidase activity,
(●) Sulfatase activity.
0.2 M Citrate-phosphate buffer (below pH 7.5) and 0.2 M tris-HCl buffer (above pH 7.5) were used.

TABLE I. SUMMARY OF PARTIAL PURIFICATION OF FUNGOUS MYROSINASE

Treatment	Activity (μmoles/min/ml)	Sp. activity (μmoles/min/mg)	Protein (mg/ml)	Volume (ml)	Yield (%)
Broth	0.08	0.065	1.20	5000	100
Precipitation with 0.9 satn. of $(NH_4)_2SO_4$	0.31	0.093	3.24	1100	84.4
Adsorption on DEAE-cellulose	1.36	0.79	1.72	150	52.0
Chromatography on DEAE-cellulose	4.14	2.93	1.40	30	31.6
Rechromatography on DEAE-Sephadex	2.06	10.07	0.21	41	21.6

The enzymatic activities were measured by the liberation of glucose.

sulfatase was made by chromatography on a DEAE-Sephadex column. The purified enzyme was chromatographed on a DEAE-Sephadex column equilibrated with 0.01 M phosphate buffer, pH 7.0. Elution was performed with a linear gradient from 0 to 0.2 M sodium chloride in the same buffer. As shown in Fig. 8, the activity peaks of thioglucosidase and sulfatase appeared at the same position on the chromatogram, and the ratio of the two activities was unity across this peak. Therefore, it was confirmed to be impossible to distinguish the two activities chromatographically.

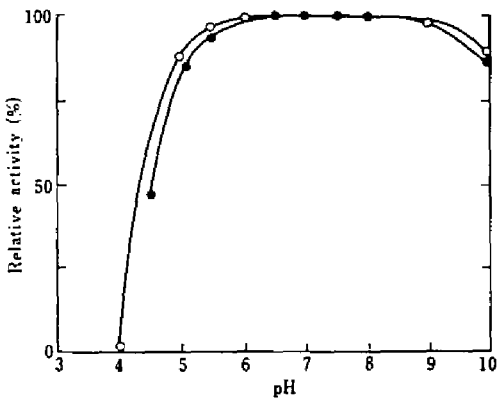


FIG. 5. pH-Stability Curves of the Fungous Myrosinase.

The sample solution was incubated in 0.005 M citrate-phosphate buffer (below pH 7) and 0.005 M tris-HCl buffer (above pH 7) for 30 min at 37°C before measurement of the enzymatic activities.
(○) Thioglucosidase activity,
(●) Sulfatase activity.

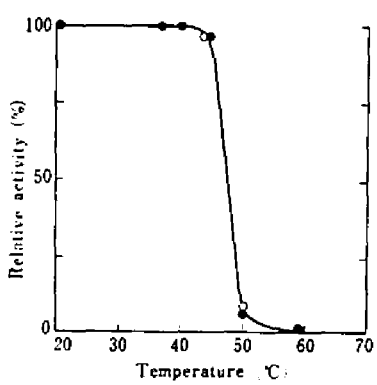


FIG. 6. Temperature-Stability Curves of the Fungous Myrosinase.

The sample solution was incubated in 0.01 M phosphate buffer, pH 7.0 for 15 min at each temperature before measurement of the enzymatic activities.
(○) Thioglucosidase activity,
(●) Sulfatase activity.

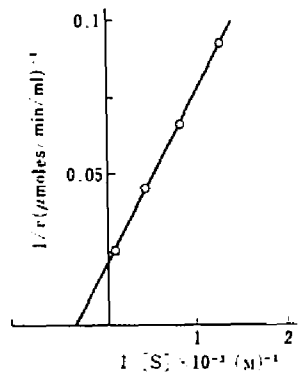


FIG. 7. Lineweaver-Burk Plot of Fungous Myrosinase in Sinigrin Hydrolysis.

The enzymatic activities were measured by the liberation of sulfate.

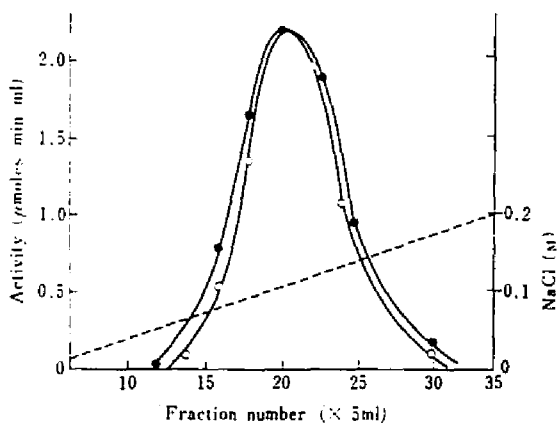


FIG. 8. Chromatogram of the Purified Fungous Myrosinase on a DEAE-Sephadex Column (1×15 cm).
(●) Thioglucosidase activity,
(○) Sulfatase activity,
(—) NaCl conc.

DISCUSSION

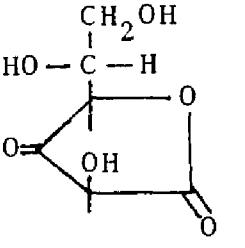
A culturing medium for the production of the fungous myrosinase by *Aspergillus sydowi* was proposed, where the enzyme could be effectively secreted by constant maintenance of broth in the neutral pH range.

The fungous myrosinase was purified approximately 150 fold. Although its hydrolyzing activity on the substrate was measured by its thioglucosidase and sulfatase activities, these two activities showed good agreement in the pH-activity, pH-stability and temperature-stability curves. The chromatographic behaviors of the two activities were not distinguishable. These findings suggest that the fungous myrosinase is not a mixture of the two enzymes, thioglucosidase and sulfatase.

It was inferred by Ettlinger and Lundeen[4] and by Nagashima and Uchiyama[5] that the thioglucoside bond of mustard oil glucoside was cleaved by the attack of plant myrosinase (β-thioglucosidase) resulting in the spontaneous release of sulfate. In accordance with the Ettlinger-Nagashima opinion,[4,5] the author conclude that the myrosinase produced by *Aspergillus sydowi* is—similar to plant myrosinase—not a mixture of thioglucosidase and sulfatase but a single β-thioglucosidase. Further experimental results supporting this conclusion will be presented in later Chapters.

Optimum pH range of the fungous myrosinase was limited to about pH 7.0 as compared with that of the plant myrosinase[7,13] with a range of pH 5~7. The fungous myrosinase was less stable than that of the plant myrosinase[7,13] in the acidic pH range and at high temperatures (Figs. 4, 5 and 6).

The *Km* value of the fungous myrosinase was larger by about 20 fold than that of the non-activated plant myrosinase.[7] The affinity of the fungous myrosinase to the substrate, sinigrin, is considerably less than that of the plant enzyme,[7] so far as judged from the *Km* values.



Ascorbic acid
(one keto form)

Chapter 2

Effects of Various Reagents

In the preceding Chapter, several characteristics of fungous myrosinase were described and I concluded that this fungous myrosinase was, similar to plant myrosinase,[4~7] a β-thioglucosidase in nature.

The effects of several reagents on fungous myrosinase were previously reported by Reese *et al.*[1] and by Kojima and Tamiya.[2] While, inhibitioh by neutral salts and competitive inhibition by sugars and β-glucosides on plant myrosinase were described by Tsuruo and Hata.[14,15] The effect of neutral salts on fungous myrosinase has never been reported and any inhibitory effects of the β-glucosides on it was denied by Reese *et al.*[1]

In this Chapter, I described the effects of various reagents including neutral salts, sugars and glucosides on fungous myrosinase in comparison with their effects on plant myrosinase.

RESULTS

Effects of various reagents on fungous myrosinase

Effects of various reagents were tested. Cobalt (II), zinc (II) and magnesium ions stimulated enzyme activity at 10^{-3} M, whereas mercury (II), iron (II) and copper (II) ions inhibited it (Table I). *p*-Mercuribenzoate, iodoacetate, diisopropylfluorophosphate, N-ethylmaleimide, ethylenediaminetetraacetate and *o*-phenanthroline were without effects (Table II). δ-Gluconolactone, a specific inhibitor of β-glucosidases,[16] strongly inhibited the enzyme activity.

TABLE I. EFFECT OF INORGANIC SALTS ON FUNGOUS MYROSINASE

Inorganic salts	Relative activity (%)
None	100
$CoCl_2$	145
$ZnCl_2$	116
$CaCl_2$	106
$MgCl_2$	111
$SrCl_2$	100
$CuCl_2$	75
$HgCl_2$	19
$NiSO_4$	105
$FeSO_4$	58
LiCl	99

All inorganic salts were tested at 10^{-3} M.
Reactions were carried out at pH 7.0 and 37°C.

TABLE II. EFFECT OF VARIOUS REAGENTS ON FUNGOUS MYROSINASE

Reagents	Conc. (M)	Relative activity (%)
None		100
p-Mercuribenzoate	2×10^{-4}	95
Iodoacetate	2×10^{-3}	102
N-Ethylmaleimide	2×10^{-3}	104
Ethylenediamine-tetraacetate	10^{-3}	93
o-Phenanthroline	10^{-3}	92
Diisopropylfluoro-phosphate	3×10^{-3}	100
δ-Gluconolactone	10^{-2}	32
Pottasium iodide	10^{-3}	100

Effect of ascorbate

Characteristic effects of L-ascorbate on plant myrosinase were emphasized by Tsuruo and Hata.[17] That is, plant myrosinase is activated by ascorbate[18,19] at concentrations between 10^{-5} and 5×10^{-3} M and is inhibited at concentrations higher than 5×10^{-3} M.[17] The

effect of L-ascorbate on fungous myrosinase is shown in Fig. 1. The fungous myrosinase

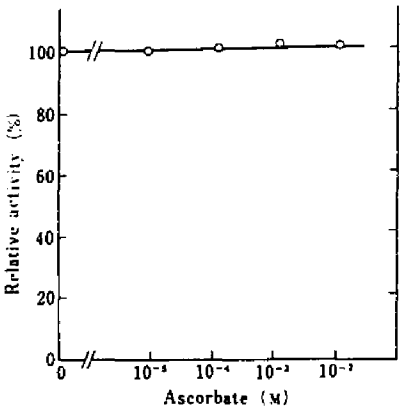


FIG. 1. Effect of Ascorbate on Fungous Myrosinase.
Reactions were carried out at pH 7.0 and 37°C.

was neither activated nor inhibited at any concentrations of ascorbate.

Effects of sodium chloride

Fungous myrosinase was inhibited by sodium chloride at high concentrations. The relationship between concentration of sodium chloride and reaction rate is shown in Fig. 2.

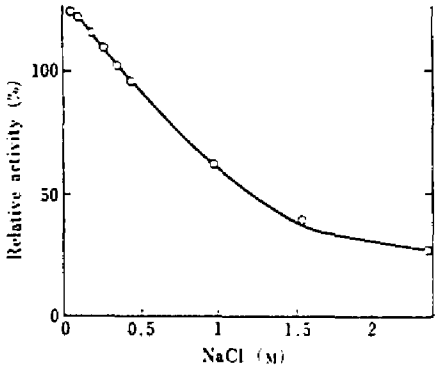


FIG. 2. Effect of NaCl on Fungous Myrosinase.
Enzymatic reactions were carried out at pH 7.0 and 37°C.

Effects of sugars and glucosides

Effects of sugars and glucosides on fungous myrosinase were tested. Among the sugars, glucose inhibited the enzyme (Table III). All glucosides tested inhibited it (Table IV). Inhibition by amygdalin was the strongest.

TABLE III. EFFECT OF VARIOUS SUGARS ON FUNGOUS MYROSINASE

Sugars	Relative activity (%)
None	100
Glucose	49
Galactose	95
Mannose	100
Xylose	103
Fructose	102
Maltose	90
Sucrose	104
Cellobiose	91

All sugars were tested at 10^{-1} M.
Reactions were carried out at pH 7.0 and 37°C.

TABLE IV. EFFECT OF VARIOUS GLUCOSIDES ON FUNGOUS MYROSINASE

Glucosides	Relative activity (%)
None	100
Salicin	78
Amygdalin	2
Arbutin	73
α-Methylglucoside	75
β-Methylglucoside	79
β-Phenylglucoside	48
p-Nitrophenyl β-glucoside	69

p-Nitrophenyl β-glucoside was tested at 5×10^{-2} M; others at 10^{-1} M.
Reactions were carried out at pH 7.0 and 37°C.

The inhibitory effects of glucose and salicin are demonstrated as Lineweaver-Burk plots in Fig. 3, plotted in the absence and presence of glucose or salicin. Evidently, the inhibition by glucose and salicin are competitive in type. The *Ki* values for glucose and for salicin were calculated to be 2.2×10^{-2} M and 1.4×10^{-1} M, respectively.

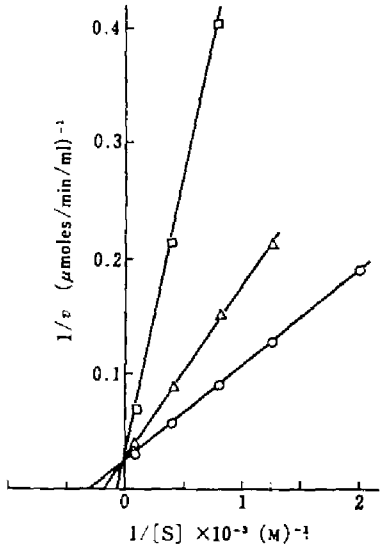


FIG. 3. Inhibitory Effect of Glucose and Salicin on Sinigrin Hydrolysis.

Enzymatic reactions were carried out with 10^{-1} M glucose (□) or salicin (△) and without them (○) at pH 7.0 and 37°C.

DISCUSSION

Effects of various reagents on this fungous myrosinase were investigated. The fungous myrosinase was stimulated by cobalt (II), zinc (II) and magnesium ions and was inhibited by mercury (II), iron (II) and copper (II) ions, but metal-complexing agents (ethylenediaminetetraacetate and *o*-phenanthroline) were without effects (Tables I and II). Although plant myrosinase is an SH-enzyme,[18] fungous myrosinase was not inhibited by *p*-mercuribenzoate, iodoacetate and N-ethylmaleimide. Therefore, sulfhydryl groups were probably not required for the enzymatic activity of this enzyme. Also, as diisopropylfluorophosphate did not inhibit enzymatic activity, serine residues were probably not required for this activity. A fungous myrosinase prepared by Reese *et al.*[1] from a culture broth of the same fungus, *Aspergillus sydowi*, was reported to be inhibited by cobalt (II) chloride and *p*-mercuribenzoate. These phenomena do not agree with the results presented here. However, results obtained by Kojima and Tamiya[21] with an enzyme preparation from the same fungus agree with the present results. Perhaps, there may be two types of myrosinase secreted by *Aspergillus sydowi*; the one studied by Kojima and Tamiya[21] and the present author and another which was studied by Reese *et al.*[1]

Aspergillus sydowi myrosinase was neither activated nor inhibited by any concentrations of L-ascorbate (Fig. 1) in spite of its characteristic effects[17~19] on plant myrosinase. Oginsky *et al.*[3] reported that bacterial myrosinase of *Paracolobactrum aerogenoides* was inhibited by 5 mg/ml ascorbate. The effect of ascorbate appears to be in remarkable contrast between fungous and plant myrosinases.

Fungous myrosinase was inhibited by high concentrations of sodium chloride (Fig. 2). The inhibition behavior of sodium chloride shown in this figure resembles that of ascorbate-activated plant myrosinase.[20] It also resembles inhibition of fungous β-glucosidase as studied by Jermyn.[21] In order to detect the effect of ionic strength on this enzyme, data presented in Fig. 2 were plotted as common logarithms of enzymatic activities against the square roots of the ionic strength of sodium chloride[20] (Fig. 4). A straight line

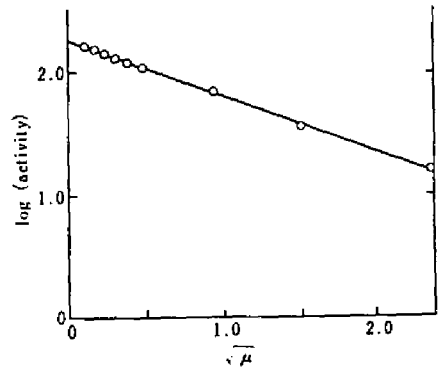


FIG. 4. Relationship of the Ionic Strength of NaCl to Fungous Myrosinase Activity.

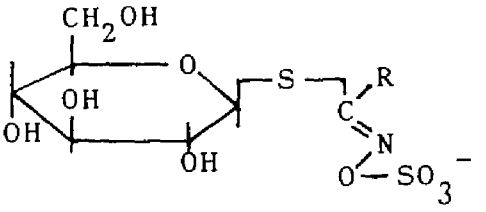
μ=Ionic strength.
Data presented in Fig. 2 are plotted.

with negative slope was obtained. Hence, the effect of sodium chloride on the enzyme is considered to be an effect of the ionic strength of the solution.[20,22]

Reese *et al.*[1] reported that possible competitive inhibitors including several β-glucosides had no effects on fungous myrosinase. Their report does not agree with the experimental results given in this paper. The reason for this inconsistancy is presumably ascribable to the low concenrations of the β-glucosides used by Reese *et al.*

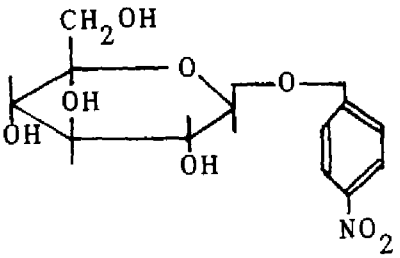
β-Glucosidases of almond,[23] fungous[24] and yeast[25] origins are competitively inhibited by sugars and β-glucosides. The conclusion that inhibition by glucose and salicin are competitive in type (Fig. 3) suggests, similar to plant myrosinase,[14] a strong resemblance of fungous myrosinase to the β-glucosidases. This resemblance is also supported by the fact that the enzyme was inhibited by δ-gluconolactone[11] (Table II). The conclusion presented in the previous Chapter, that fungous myrosinase is not a mixture of thioglucosidase and sulfatase, but a single β-thioglucosidase is again supportable by these results. In the present Chapter, enzymatic activities were measured by liberation of sulfate, *i.e.*, they were expressed as "sulfatase" activities. But fungous myrosinase was competitively inhibited by glucose and salicin. Therefore, this supports a degradation mechanism of mustard oil glucoside as proposed by Nagashima and Uchiyama,[5] where the liberation of sulfate is the result of hydrolytic cleavage of the thioglucoside bond of the glucoside by a single thioglucosidase followed by spontaneous cleavage of the N-O bond.

The fact that fungous myrosinase was inhibited competitively by sugars and β-glucosides suggests that it would hydrolyze β-glucoside substrate in a way similar to plant myrosinase.[17]



Mustard oil glucoside

Sinigrin: R- = CH_2=CH-CH_2-



p-Nitrophenyl β-glucoside

Chapter 3

On the β-Glucosidase Activity

In a previous Chapter, the fungous myrosinase produced by *Aspergillus sydowi* was concluded to be a β-thioglucosidase as is plant myrosinase.[4~7] This conclusion was also supported by the fact that the enzyme was competitively inhibited by glucose and β-glucosides.

Competitive inhibition by sugars and β-glucosides of plant myrosinase was described by Tsuruo and Hata,[14] and the possession of β-glucosidase activity by plant myrosinase was confirmed by these same authors[17] in spite of the belief of earlier years[26] that the enzyme had a strict substrate specificity for mustard oil glucoside. Resemblance of plant myrosinase to the β-glucosidases has also been pointed out.[14,17]

Previously, Reese *et al.*[1] reported the hydrolysis of *p*-nitrophenyl β-glucoside (*p*-NPG) and *o*-nitrophenyl β-galactoside by a crude preparation of *Aspergillus sydowi* myrosinase. From the results[14,17] obtained with the plant myrosinase, purified fungous myrosinase was definitely thought to have β-glucosidase activity. Investigation on the β-glucosidase activity of this enzyme contributes to knowledge about the relationship between myrosinases and β-glucosidases. Some results of studies in this area are presented here.

MATERIALS AND METHODS

Enzyme preparation. The previously described method of preparing the enzyme solution was improved as follows.

Aspergillus sydowi IFO 4284 was cultured on the liquid medium described previously under shaking at 29°C for 2 weeks. After cultivation, the broth was filtered and the enzyme was precipitated from this filtrate by bringing ammonium sulfate concentration to 90% of saturation. The precipitate was dissolved in 0.01 M phosphate buffer, pH 7.0 and dialyzed against the same buffer. The dialyzate was mixed with 60 g (dry matter) of DEAE-cellulose powder in the same buffer and adsorbed protein was eluted by 0.1 M sodium chloride in 0.01 M phosphate buffer, pH 7.0. The eluted protein was precipitated by 90% of saturation of ammonium sulfate. The resulting precipitate was dissolved in 0.01 M phosphate buffer, pH 7.0 and dialyzed against the same buffer. (Hereafter, in purification procedures, the concentration of the enzyme solution was performed by ammonium sulfate precipitation as described above.) The concentrate was charged on a DEAE-Sephadex column (300 ml, *i.d.* 4 cm), which was equilibrated with the same buffer and adsorbed protein was eluted by a linear gradient of 0~0.2 M sodium chloride. After concentrating the eluate, gel-filtration on Sephadex G-200 column (450 ml, *i.d.* 2.5 cm) was carried out. The filtrate was then charged on a DEAE-Sephadex column (15 ml, *i.d.* 1 cm), equilibrated with the same buffer and adsorbed protein was eluted by a linear gradient of 0~0.2 M sodium chloride. After concentrating this eluate, gel-filtration on a combined column[27] of Sephadex G-100 (120 ml, *i.d.* 1.5 cm; down flow) and G-150 (200 ml, *i.d.* 2.5 cm; up flow) was performed. This filtrate was used throughout the experiment as the purified enzyme preparation. A summary of the purification procedures of the

TABLE I. SUMMARY OF PURIFICATION OF FUNGOUS MYROSINASE

Treatment	Step	Activity (μmoles/min/ml)		Specific activity (μmoles/min/mg)		Protein (mg/ml)
		Sinigrin hydrolysis	*p*-NPG hydrolysis	Sinigrin hydrolysis	*p*-NPG hydrolysis	
Culture broth	A	0.12	0.05	0.26	0.10	0.48
Ppt. with 0.9 satn. of $(NH_4)_2SO_4$	B	1.09	0.42	0.32	0.12	3.46
Adsorption on DEAE-cellulose	C	4.90	1.87	0.97	0.37	5.06
Chromatography on DEAE-Sephadex	D	5.10	0.36	12.1	0.85	0.42
Gel-filtration on Sephadex G-200	E	18.1	1.40	68.0	5.26	0.27
Chromatography on DEAE-Sephadex	F	24.8	1.14	114	5.26	0.22
Gel-filtration on a Sephadex G-100 and G-150 combined column	G	19.4	0.94	111	5.26	0.17

enzyme is shown in Table I.

Assay of sinigrin hydrolysis. The assay mixture contained 2.5 μmoles of sinigrin and the enzyme in a total volume of 1 ml. Hydrolytic rates were measured by titration of liberated sulfate using a recording pH-stat, Radiometer Model SBR2/SBU1/TTT1 Auto-titrator, as described previously.[1] Enzymatic reactions were carried out at pH 7.0 and 37°C under conditions regarded to be zero order reactions.

Assay of p-NPG hydrolysis. The assay mixture contained 1 μmole of *p*-NPG, 0.1 mmole of phosphate buffer, pH 7.0 and the enzyme in a total volume of 1 ml. Reactions were carried out at 37°C under conditions regarded to be zero order reactions. After the reaction was over, 5 ml of 8% aqueous dipotassium hydrogen phosphate solution was added to the reaction mixture and liberated *p*-nitrophenol was measured colorimetrically at 400 mμ using a Shimadzu Model QV-50 spectrophotometer.

Assay of other glucoside hydrolyses. The assay mixture contained 4 μmoles of each glucoside, 0.1 mmole of phosphate buffer, pH 7.0 and the enzyme in a total volume of 1 ml. Enzymatic reactions were carried out at 37°C.

Rate of amygdalin hyrolysis was measured by determination of liberated cyanide using the method of Alridge.[28]

Rate of cellobiose hydrolysis was measured by determination of liberated glucose using the method of Tauber and Kleiner.[29]

Cellulose acetate membrane electrophoresis. Sample solutions containing 30 μg of protein in 0.05 M phosphate buffer, pH 8.5 or 0.07 M veronal buffer, pH 8.6 were spread on Oxoid cellulose acetate membranes (Oxo Ltd.) 2.2 cm in width. Electrophoretical experiments were carried out in the same buffers at 0°C with 0.8 mA/cm for 30 min. After electrophoresis, the distribution of protein on the membrane was shown by staining with Ponseau 3R.

RESULTS

The myrosinase solution obtained from the culture broth of *Aspergillus sydowi* possessed remarkable β-glucosidase activity. As shown in Fig. 1, color development of *p*-nitrophenol after incubation of the enzyme preparation with *p*-NPG was observed. The enzyme solution had hydrolytic activity toward various β-glucosides (Table II). Its *Km* value for *p*-NPG was calculated to be 1.0×10^{-4} M. (The Lineweaver-Burk plot for *p*-NPG hydrolysis: see Fig. 4.)

The wide substrate specificity on β-glucoside substrate (Table II) raised a doubt as to whether the enzyme solution had been contaminated by β-glucosidase secreted by the fungus in the original culture broth. The possibility of this contamination is, indeed, shown in Table I, where *p*-NPG hydrolytic activity is tabulated together with sinigrin hydrolytic activity. Apparently, the ratios of specific activities of sinigrin hydrolysis to *p*-NPG hydrolysis increased in parallel with the purification steps. This fact indicates that there were β-glucosidases other than myrosinase itself in the culture broth. But,

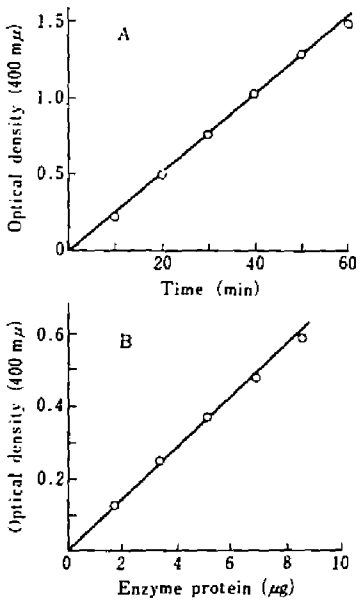


FIG. 1. Fungous Myrosinase and *p*-NPG Hydrolysis.

A. Time course of *p*-NPG hydrolysis. Ten μl of enzyme solution was used.

B. Enzyme concentration and *p*-NPG hydrolysis (Reaction time; 6 min).

Reactions were carried out at pH 7.0 and 37°C.

TABLE II. SUBSTRATE SPECIFICITY OF FUNGOUS MYROSINASE

Substrate	Hydrolytic rate (μmoles/min/ml)
Sinigrin	9.55
Salicin	0.21
Amygdalin	trace
Arbutin	1.14
Cellobiose	1.12
α-Methylglucoside	0
β-Methylglucoside	0.19
β-Phenylglucoside	0.78
p-Nitrophenyl β-glucoside	0.65

The hydrolytic rates were determined at 4 mM for each substrate. Reactions were carried out at pH 7.0 and 37°C.

in the last two purification steps (Steps F and G), both specific activities reached constant values. Chromatographic uniformity of the enzyme preparation in the present experiment is shown in Fig. 2. This figure also shows

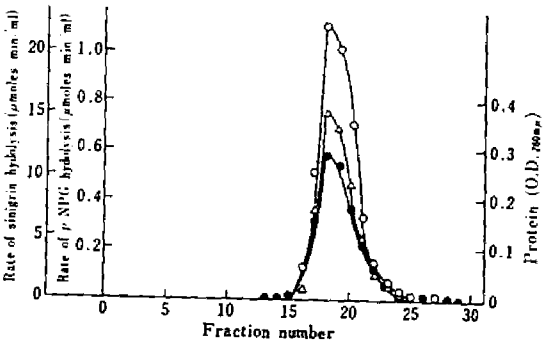


FIG. 2. Gel-filtration Pattern of Partially Purified Fungous Myrosinase on a Sephadex G-100 and G-150 Combined Column.

Protein (7.7 mg) was contained in the sample solution. Gel-filtration was performed in 0.01 M phosphate buffer, pH 7.0. The fraction was 5 ml.

Sinigrin hydrolysis ○—○, *p*-NPG hydrolysis △—△, protein ●—●.

an agreement of hydrolytic activities for sinigrin and *p*-NPG on the chromatogram. Uniformity of the enzyme preparation was also detected by electrophoretic studies on cellulose acetate membrane (Fig. 3). These results indicate that contamination of this fungous myrosinase preparation by β-glucosidase did not occur.

In order to prove that *p*-NPG was hydrolyzed by catalytic action of the fungous myrosinase itself, as described by Levvy and Marsh,[30] the inhibitory effect of the specific substrate, sinigrin, on *p*-NPG hydrolysis was tested. Results are shown in Fig. 4. It is evident from this figure that *p*-NPG hydrolysis was inhibited competitively by sinigrin. Deviations of plots from a straight line in the presence of inhibitor at low concentrations of *p*-NPG are due to hydrolysis of the inhibitor itself. The *Ki* value for sinigrin was calculated to be 3.8×10^{-3} M and this value is in good agreement with the *Km* value for sinigrin (3.6×10^{-3} M) of the enzyme. Thus, *p*-NPG hydrolytic activity of fungous myrosinase is proved.

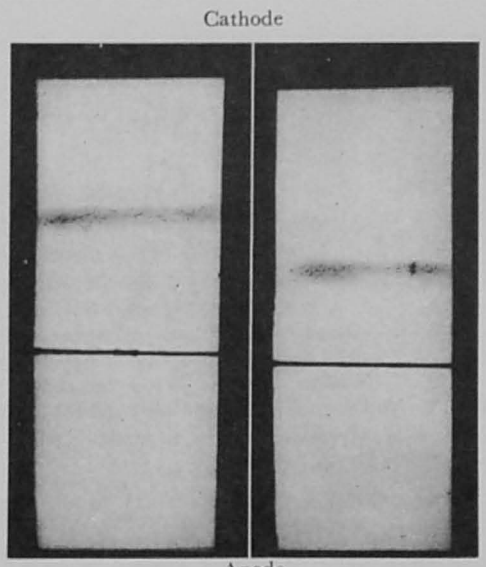


FIG. 3. Electrophoretic Patterns of Fungous Myrosinase on Cellulose Acetate Membrane.

Electrophoretic experiments were carried out on Oxoid cellulose acetate membrane at 0°C with 0.8 mA/cm for 30 min. Protein distribution was shown by staining with Ponseau 3R
Left; in 0.07 M veronal buffer, pH 8.6.
Right; in 0.05 M phosphate buffer, pH 8.5.

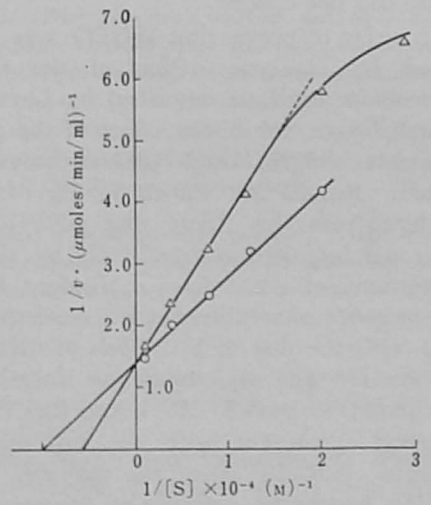


FIG. 4. Inhibitory Effect of Sinigrin on *p*-NPG Hydrolysis.

Reactions were carried out in the presence (△) and absence (○) of 2.5 mM sinigrin at pH 7.0 and 37°C.

DISCUSSION

Reese *et al.* [1] studied the substrate specificity of *Aspergillus sydowi* myrosinase using its crude preparation and reported hydrolyses of various glucosides. As for plant myrosinase, its hydrolytic activity on *p*-NPG and *o*-nitrophenyl β-glactoside was pointed out by Reese *et al.* [1] and was later studied in detail by Tsuruo and Hata.[17] In the present paper, the β-glucosidase activity of fungous myrosinase was affirmed. A problem of what relationship exists between myrosinases and β-glucosidases occurs.

Previously, a widely accepted view on plant myrosinase, according to Veibel's description,[26] was that it possesses very pronounced specificity and hydrolyzes only naturally occurring thioglucosides, *i.e.* mustard oil glucosides, but not synthetically prepared thioglucosides. On the other hand, it was believed[17,31] that neither mustard oil glucosides nor synthetic thioglucosides were hydrolyzable by other known glycosidases. Since the thioglucoside bond is more resistant against acid-catalyzed hydrolysis than is the glucoside bond,[32] the former bond would be more stable than the latter are when they combine identical aglycon. Accordingly, a thioglucoside bond-cleaving enzyme must be strictly specialized for its substrate. Partly from this assumption, hydrolysis of natural and synthetic β-tnioglucosides by β-glucosidases was not believed to be possible at the time when Veibel's review[26] appeared. Conversely, it has become hence explanable that enzymatic hydrolyses of mustard oil glucosides require the specialized enzyme, myrosinase, and the presence of electron-attracting sulfonic acid group[3] in aglycon moieties of the thioglucosides. Uchiyama[33] interpreted the observation of Reese *et al.*, [1] that *p*-NPG and *o*-nitrophenyl β-galactoside were hydrolyzed by plant and fungous myrosinases but phenyl β-thioglucoside and 4-methoxyphenyl β-thioglucoside were not. Uchiyama's interpretation for this observation was that the former two compounds had an electron-attracting nitro group in their aglycon moieties but the latter two had no electron-attracting group. This interpretation supports the above assumption. Other observations

made by Reese *et al.*[1] and by Nagashima and Uchiyama,[3] that desulfonated mustard oil glucosides, S-β-D-1-glucopyranosylphenylacetothiohydroxamic acid and merosinigrin, were not hydrolyzed (Nagashima) or only slightly hydrolyzed (Uchiyama) also lend support to the above assumption.

However, hydrolysis of phenyl β-thioglucoside by *Stachybotrys atra* β-glucosidase was reported by Jermyn[34] and hydrolysis of 6-purinyl β-thioglucoside by almond emulsin was reported by Goodman *et al.*[35,36] Hydrolyses of *p*- and *o*-phenyl β-thioglucosides by almond emulsin were also described by Wagner and Metzner.[37] In contrast, hydrolyses of some β-thioglucosides including 6-purinyl β-thioglucoside and 2,4-dinitrophenyl β-thioglucoside by plant myrosinase were reported by Goodman *et al.*[38,39] In addition, the wide distribution of the β-thioglucosidase which hydrolyzes 6-purinyl β-thioglucoside in higher animals was shown by Goodman *et al.*[38] As for plant and fungous myrosinases, the description of Reese *et al.*[1] of the hydrolyses of *p*-NPG and *o*-nitrophenyl β-galactoside was refered earlier in this paper. Tsuruo and Hata[14,17] pointed out close relationship of plant myrosinase to the β-glucosidases based on experimental results from inhibition by sugars and β-glucosides and the hydrolysis of *p*-NPG. The present paper has shown the result of studies on *p*-NPG hydrolysis by fungous myrosinase.

All reports cited show that the distinctions between myrosinases and β-glucosidases are not so strict as previously believed,[28] and rather a close relationship exists between them. This relationship is also supported by the data on competitive inhibition by sugars and β-glucosides on plant[14] and fungous myrosinases and, conversely, by the competitive inhibition with phenyl β-thioglucoside and other aromatic β-thioglucosides on fungous[34,39] and yeast[40] β-glucosidases.

In conclusion, plant and fungous myrosinases can be rather regarded to be β-glucosidases highly specialized for hydrolyses of mustard oil glucosides, than that they are enzymes having strict substrate specificity for mustard oil glucosides. In an earlier study by Pigman[41] on the classification of carbohydrases, β-thioglucosidase of plant myrosinase was regarded as belonging to the β-glucosidase family. Nagashima and Uchiyama[3] also stated that plant myrosinase is a member of the glucosidases. These opinions are affirmed by the conclusions presented here.

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Chapter 4

Production and Stability of the Intracellular Myrosinase from Aspergillus niger

In previous Chapters,[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, I 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 characteristics 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 $MgSO_4 \cdot 7H_2O$), 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 $MgSO_4 \cdot 7H_2O$), 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, 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 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, 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.

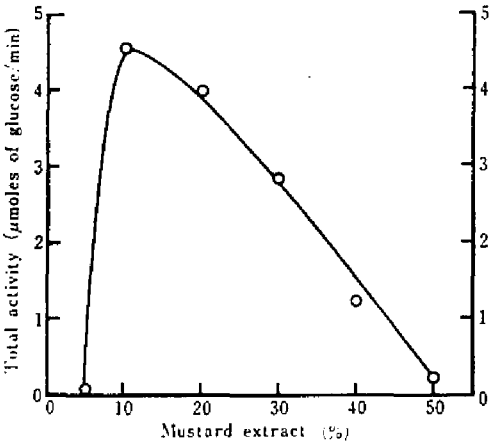

FIG. 2. Effect of Mustard Extract Concentration of Enzyme Production in Mycelia of *Aspergillus niger*.

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.

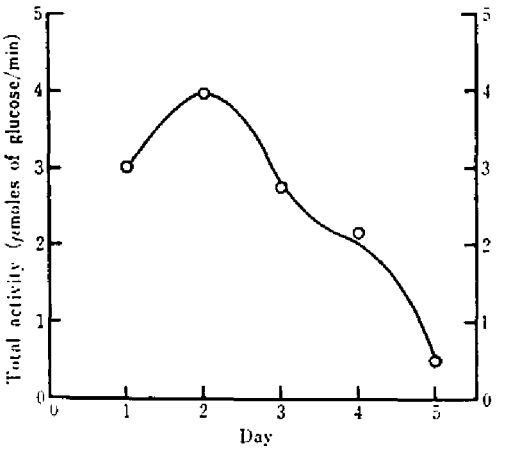

FIG. 1. Enzyme Production in Mycelia of *Aspergillus niger*.

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% $(NH_4)_2SO_4$, 0.03% $NaNO_3$ and 0.03% $MgSO_4 \cdot 7H_2O$, 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.

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% $(NH_4)_2SO_4$ and 0.05% $MgSO_4 \cdot 7H_2O$, pH 6.5.
Fungi were cultivated for 2 weeks and bacteria for 2 days at 29°C under shaking.

	Growth	Myrosinase activity
Pseudomonas aeruginosa (IFO 3080)	+++	−
Sarcina lutea (IAM 1099)	+++	−
Staphylococcus aureus (IFO 3061)	−	−
Flavobacterium fuscus (AKU 0140)	−	−
Micrococcus lysodeikticus (IFO 3333)	+	−
Alcalygenes faecalis (IAM B-141-1)	−	−
Proteus vulgaris (IFO 3167)	−	−
Serratia marcescens (IFO 3046)	±	−
Aerobacter aerogenes (IFO 3320)	+++	−
Escherichia coli (AKU 0001)	−	−
Bacterium cadaveris (IFO 3731)	±	−
Bacillus subtilis (IFO 3026)	++	−
Streptococcus faecalis (IFO 3181)	−	−
Candida utilis (IFO 0396)	−++	−
Aspergillus		
oryzae (AKU 3301)	−±	−
niger (AKU 3302)	−−+	++
fumigatus (IFO 4040)	−+	−
flavus (IFO 5839)	++	−
sydowi (IFO 4284)	−+	−
Penicillium		
notatum (IFO 4640)	−±±	−
urticae (IFP 7011)	−−+	−
Mucor rouzianus (IFO 5773)	+	−
Phizopus oryzae (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 $MgSO_4 \cdot 7H_2O$, 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
Aspergillus niger	IFO 4034	++
〃	IFO 4068	++
〃	IFO 4280	+
〃	IFO 6341	+
〃	IFO 5374	++
〃	AKU 3302	++

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 $CoCl_2 \cdot 6H_2O$) 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% $(NH_4)_2SO_4$, 0.03% $NaNO_3$ and $MgSO_4 \cdot 7H_2O$, 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% $CoCl_2 \cdot 6H_2O$ 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% $(NH_4)_2SO_4$, 0.03% $NANO_3$ and $MgSO_4 \cdot 7H_2O$, 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% $CoCl_2 \cdot 6H_2O$ 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, I 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 myrosinase[1] 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. I searched for myrosinase in various microbial mycelia in order to obtain it in large amounts. I 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

	Conc. (M) / Time (hr)	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
	24 (5°C)	72	80	91	97	45	57	98 (80)[a]
	48 (20°C)	33	34	51	65	24	12	80 (52)[a]

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

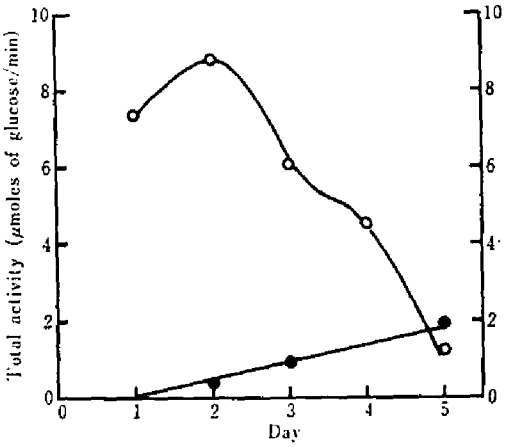

FIG. 3. Comparison of Enzyme Production by *Aspergillus niger* and *Aspergillus sydowi*.

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% $(NH_4)_2SO_4$, 0.03% $NaNO_3$ and $MgSO_4 \cdot 7H_2O$, 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% $CoCl_2 \cdot 6H_2O$, 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% $(NH_4)_2SO_4$, 0.03% $NaNO_3$, 0.03% $MgSO_4 \cdot 7H_2O$ 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 allylisothiocyanate 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 unstalbe, 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.

Chapter 5

General Characteristics

In a previous Chapter,[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, I 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

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[5,6] and was used as the substrate of fungous myrosinase. *p*-Nitrophenyl β-glucoside was of an analytical grade.

Assay of sinigrin hydrolysis. The assay mixture contained 2.5 μmoles 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.[1,5] The reaction was conducted under conditions regarded to be those for zero order reactions.

Assay of p-NPG hydrolysis. The assay mixture contained 1 μmole of *p*-NPG 0.1 μmole 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 mμ 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.*[10]

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 (4.5×23 cm) which was eqilibrated 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 in Fig. 2 were pooled and concentrated by polyethylene glycol.

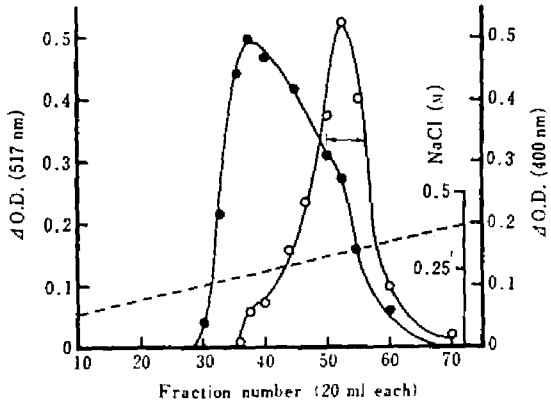

FIG. 1. Chromatogram of Fungous Myrosinase on a DEAE-Sephadex Column.

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

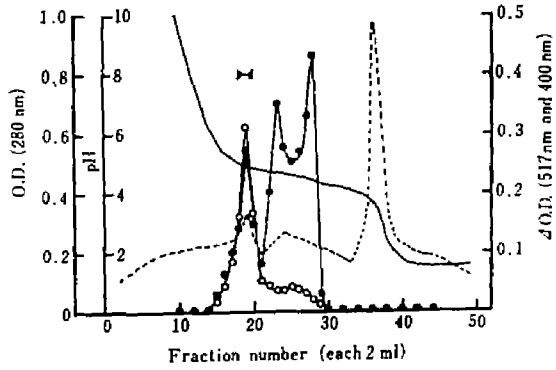

FIG. 2. Isoelectrofocusing of the Partial Purified Enzyme.

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 mμ.

Isoelectric focusing. Isoelectric focusing was carried out by the method of Vesterberg and Svensson,[11] 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, 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 fungous myrosinase is remarkably unstable.

General characteristics of the fungous 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

TABLE I. SUMMARY OF PARTIAL PURIFICATION OF FUNGOUS MYROSINASE

Treatment	Volume (ml)	Activity (μmoles/min/ml)		Specific activity (μmoles/min/mg)		Protein (mg/ml)
		Sinigrin hydrolysis	*p*-NPG hydrolysis	Sinigrin hydrolysis	*p*-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

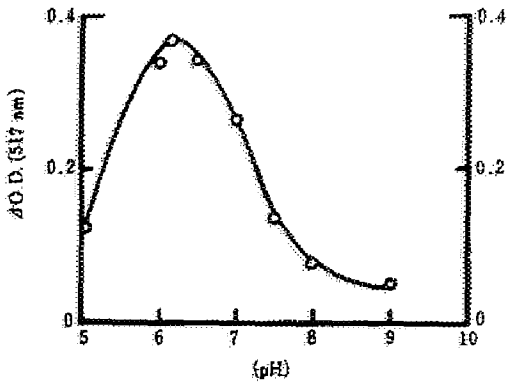

FIG. 3. pH-Activity Curve of Fungous Myrosinase.
0.2 M Sodium phosphate buffer (pH 6.0~9.0) and 0.2 M acetate buffer (pH 5.0) were used.

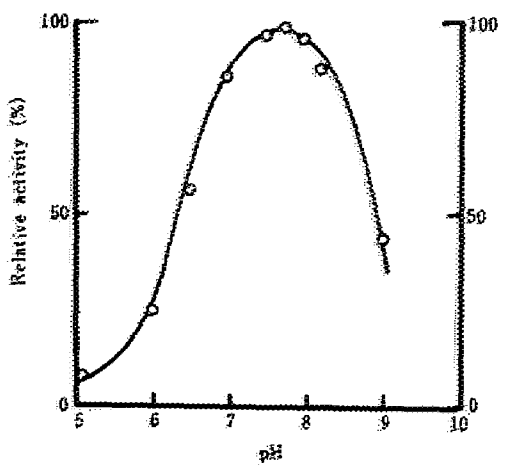

FIG. 4. pH-Stability Curve of Fungous Myrosinase.
The sample solution was incubated in 0.05 M sodium phosphate buffer (pH 6~9) and acetate buffer (pH 5.0) for 24 hr at 5°C.
Reactions were carried out at pH 6.2 and 34°C.

complexing agents showed little effect.

Plant myrosinase[9] is specifically activated by ascorbic acid (10^{-3} M), while, intracellular myrosinase was neither activated nor inhibited by L-ascrobate.

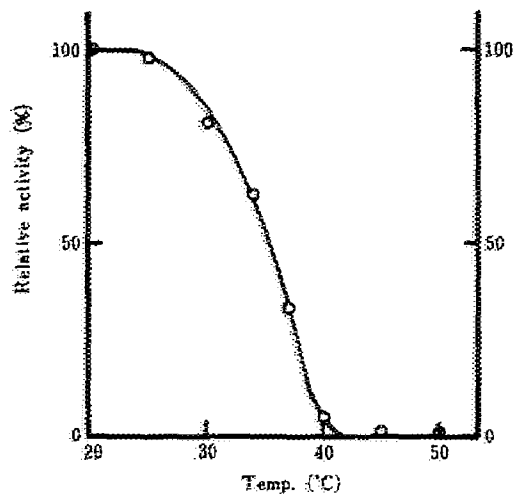

FIG. 5. Heat-stability Curve of Fungous Myrosinase.
The sample solution was incubated in 0.1 M phosphate buffer, pH 7.8 for 20 min at each temperature before measurement of enzyme activity.

δ-Gluconolactone, a specific inhibitor of β-glucosidase,[12] 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

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,[19] 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 agreement with the *Km* 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, the myrosinase produced by *Asp. niger* was remarkably unstable 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

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
o-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
p-NPG	5×10^{-2} M	106

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

	Asp. niger	*Asp. sydowi*	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+}) inhibit (Hg^{2+}, Sn^{2+})	activate (Co^{2+}) inhibit (Hg^{2+}, Fe^{2+})	—— inhibit (Hg^{2+}, Cu^{2+})
Km for Sinigrin	3.3×10^{-3} M	3.6×10^{-3} M	1.8×10^{-4} M
Ki for Sinigrin	3.8×10^{-3} M	3.8×10^{-3} M	2.0×10^{-4} M
Km for *p*-NPG	1.5×10^{-3} M	1.0×10^{-4} M	2.0×10^{-3} M

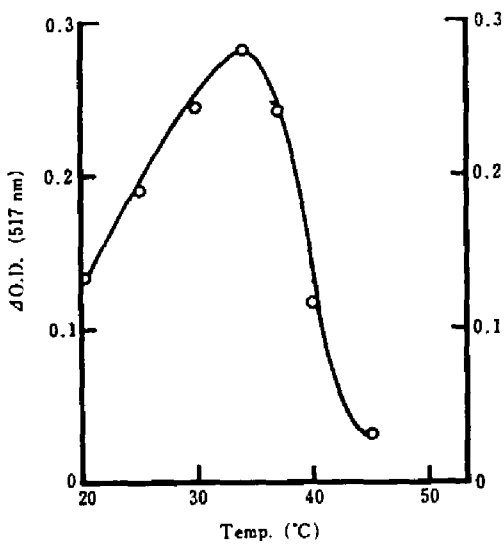

FIG. 6. Optimum-temperature Curve of Fungous Myrosinase.

Reactions were carried out at pH 6.2 for 20 min.

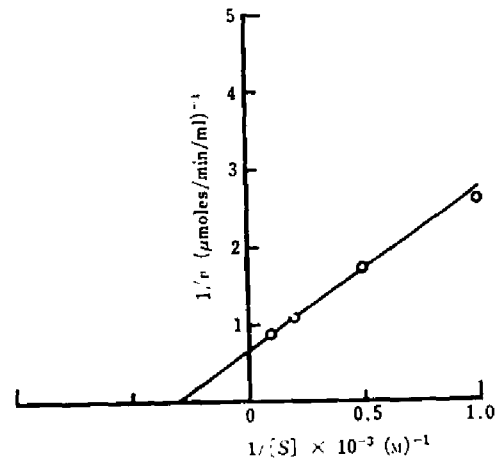

FIG. 7. Reciprocal Plot of Sinigrin Hydrolysis.

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

Km 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

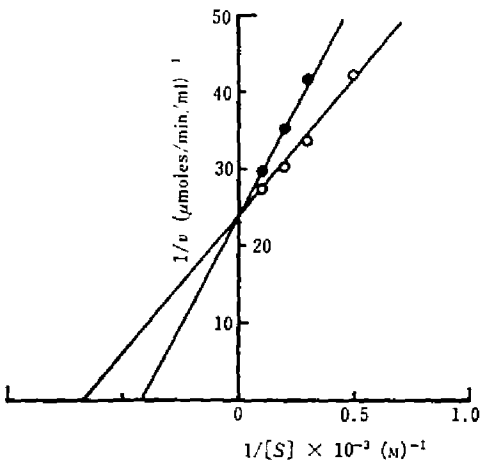

FIG. 8. Inhibitory Effect of Sinigrin on *p*-NPG Hydrolysis.

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

mustard oil glucoside. Pigman,[14] Nagashima and Uchiyama,[15,16] and Tsuruo et al. 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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PART III

Studies on the Bacterial Myrosinase

Chapter 1

Isolation of Bacteria

The fungous myrosinases were not suitable for the perpose to obtain large amounts of the stable enzyme, as one takes 2 weeks of cultivation[1] and the other is very unstable.[2]

Oginsky et al.,[3] after a screening test for myrosinase activity among intestinal bacteria, used *Paracolobactrum aerogenoides* as the active organism for their experiments. They have pointed out its existence and some properties on the crude preperation of bacterial myrosinase.

In this Chapter, I surveyed microorganism that produces myrosinase in bacteria and identified it. And I studied the optimum culture conditons for the myrosinase production and determined the purification and the general characteristics of the bacterial enzyme. The comparision of some properties of bacterial, fungous and plant myrosinases, and the relationship of the myrosinases to β-glucosidases is also discussed.

MATERIAL AND METHODS

Materials Sinigrin was obtained from Nutritional Biochemical Corporation, Ohio, U.S. Mustard powder was purchased from Amari Shokuhin, Ltd., Kyoto. All other chemicals, were obtained from commertial sources.

Organisms Almost all bacteria were kindly supplied by the Faculty of Agriculture of Kyoto University. *Paracolobactrum aerogenoides* and *Paracolobactrum coliforme* were kindly supplied by the Facultry of Agriculture of Hokkaido University. X1, X2 and X3 were isolated from sinigrin solution in my Lab..

Culture Conditions Culture conditions for screening were as follows. Microorganisms were inoculated to a test tube (1.7 x 18cm), 5 ml containing 0.1% sinigrin, 0.2% KH_2PO_4, NH_4Cl, 0.1% $MgSO_4 \cdot 7aq$ and 0.1% NaCl (pH 7.0). These cultures were cultivated at 28°C for 48 hrs.

The growth of bacteria were measured by the absorbance at 655 mμ. Decrease of sinigrin was measured by the absorbance at 228 mμ. The enzyme activity was measured by the following method.

Identification of microorganism The identification of isolated microorganism was carried out according to the description in Bergey's Manual of Determination Bacteriology, 7th edition. The medium, which was used for determination of physiological properties, was purchased from Nissin Seiyaku, Co., Ltd., Tokyo.

Enzyme preparation The cells in the culture broth were harvested by continuous flow centrifugation and were suspended with 0.2 M phosphate buffer, pH 7.0. The suspended cells were treated with a 19 KHz Kaijo-Denki ultrasonic oscillator. The cells and debris were removed by centrifugation at 10,000 r.p.m.. The resultant supernatant was used as the crude enzyme preparation.

Enzyme assay Enzymatic activities were determined as thioglucosidase and sulfatase activities, respectively. Both reactions were carried out in a system containing 2.5 μmoles of sinigrin and 0.1 μmole 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 measured by 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[4]), with a previously described modification.[5]) The data obtained were corrected by multiplying by the factor, 1.25, to eliminate the color development caused by the substrate itself.[5])

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

Assay of β-glucosidase activity The assay mixture contained 1 μmole of p-NPG, 0.1 μmole 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% KH_2PO_4 was added to the reaction mixture. Liberated p-nitrophenol was measured colorimetrically at 400 mμ.

Assay of protein Protein concentration was determined by the Lowry et al.[6])

Estimation of molecular weight The molecular weight of the enzyme was estimated by Andrew's method[7]) using a Sephadex G-100 column equilibrated with 0.01 M phosphate buffer containing 0.1 M KCl at pH 6.4. Bovine serum albumine (M.W. 67000), ovalbumine (M.W. 45000), chymotripsinogen (MW. 25000) and cytochrome C (M.W. 12400) were used as the standard protein.

Calculation of sedimentation coefficient

Sedimentation studies were carried out in a HITACHI model UCA-1A ultracentrifuge. All the runs were performed at 52,000 r.p.m. and at 20°C in a double sector cell. The S_w^{20} was calculated from series of sedimentation runs according to the conventional method.[8])

RESULTS AND DISCUSSION

Results of screening test

As shown in Table 1, the growth was observed in some strains, but the decrease of sinigrin was observed in only three strains, that is, *Arthrobactrium tumefaciens, Sarcina lutea* and X3. On these three strains, the time course of myrosinase activity was investigated. Remarkable myrosinase activity was only found in the strain X3.

Table 1. Screening of Bacteria

Strain	Growth	Decrease of Sinigrin	Myrosinase Activity	Strain	Growth	Decrease of Sinigrin	Myrosinase Activity
Escherichia coli Crookes	-	-	-	Sarcina aurantiaca IFO 3064	-	-	-
" intermedia A-21	-	-	-	" lutea IFO 1099	+	+	-
Aerobacter aerogenes IFO 3320	-	-	-	" " IFO 3232	+	+	-
" cloacae IAM 1221	+	-	-	" marginata IFO 3066	-	-	-
Serratia marcescens IFO 3054	-	-	-	" variabilis IFO 3067	-	-	-
" polymuthicum IFO 3055	-	-	-	Corynebacterium equi IAM 1035	-	-	-
Achromobacter polymorph	-	-	-	" fascians IAM 1079	-	-	-
" superficialis	-	-	-	Arthrobacter simplex IFO 3530	+	-	-
Bacillus aneurinolyticum (K.A)	-	-	-	" oxydans IFO 12138	-	-	-
" megaterium	-	-	-	" pascens IFO 12139	-	-	-
" natto Sawamura	-	-	-	Brevibacterium ammoniagenes IFO 12071	-	-	-
" mesenterium var. flavus	-	-	-	" sp. p145	-	-	-
" roseus (Migula) IAM 1257	-	-	-	Bacterium cadaveris IFO 3734	-	-	-
" subtilis IFO 3007	-	-	-	Pseudomonas fluorescens IFO 3081	-	-	-
" aphaericus IFO 3525	+	-	-	" riboflavina IFO 3140	-	-	-
" brevis IFO 3331	-	-	-	" striafaciens IFO 3309	-	-	-
" pumilus IFO 3030	-	-	-	" M4	-	-	-
" circulans IFO 3329	-	-	-	" striata	-	-	-
" licheniformis IAM 11054	-	-	-	" iodina IFO 3558	-	-	-
" aphaericus IFO 3341	-	-	-	" ovalis IFO 3736	-	-	-
Agrobacterium tumefaciens IAM B-26-1	+	+	-	Flavobacterium fuscum	-	-	-
" radiobacter IAM 1526	-	-	-	Alcaligenes faecalis	-	-	-
Micrococcus lysodeikticus IFO 3341	-	-	-	Proteus vulgaris	-	-	-
" flavus IFO 3242	-	-	-	Streptococcus faecalis	-	-	-
" luteus IFO 3763	+	-	-	Paracolobactrum aerogenoides	+	-	-
" glutamicus No.534	-	-	-	" coliforme	-	-	-
" sp. No.133	-	-	-	X 1	+	-	-
" sp. No.431	-	-	-	X 2	-	-	-
Staphylococcus aureus IFO 3060	-	-	-	X 3	++	++	++

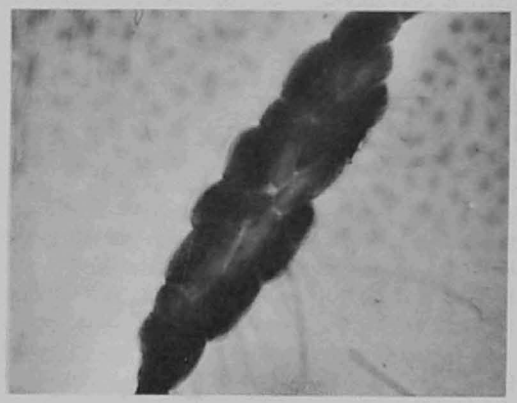


Fig. 1 Electron Micrograph of Strain X3.

Table 2 Morphological and Physiological Properties of Strain X3.

Microscopic observation (Fig. 1)
Cell; Gram negative, Rod, motile
Size of cell; 0.3 to 0.4 by 1.0 to 1.2 μ
Agar colony; circular, convex, entire, white and translucent.
Nutrient broth; turbidity, a little sediment, no ring and gas production.
1) Catalase; Positive
2) Oxidase; Negative
3) Urease; Positive
4) MR test; Negative
5) VP test; Positive
6) Indole; not produced
7) NO_3 reduction; Positive
8) NO_2 reduction; Positive
9) H_2S; not produced in nutrient broth, but produced on Christensen's medium
10) Gluconate oxidation; Positive (brown ppt.)
11) Malonate utilization; Negative
12) Phenylalanine deamination; Negative
13) Litmus milk; weakly acid, no coagulation (4 days)
14) Gelatine stab.; no liquefation (7 days)
15) Hugh-Leifson test; fermentatively acid and gas produced
16) King's medium; no fluorescence (A)
17) King's medium; no fluorescence (B)
18) Na-citrate utilization; Positive on Christensen's medium
19) Amino acid degradation;
Arginine... Positive (NH_3 reaction +)
Lysine ... Negative
Ornithine ... Positive
Glutamic acid ...Negative

Taxonomical properties of strain X3

Morphological and physiological properties of this strain are listed in Table 2 and production of gas and acid from carbohydrates is shown in Table 3. From these results, strain X3 was identified as an Enterobacter cloacae no. 506. This was used in the following experiments.

Determination of the culture conditions
Production of myrosinase

The time course of myrosinase production and decrease of sinigrin were studied. As shown in Fig. 2, sinigrin rapidly decreased, with the cultivation time and the enzyme was mostly produced at 36 hrs. This is an intracellular enzyme such as the one from *Aspergillus niger*[2] and its maximum production rate is also like that of *Asp. niger*.

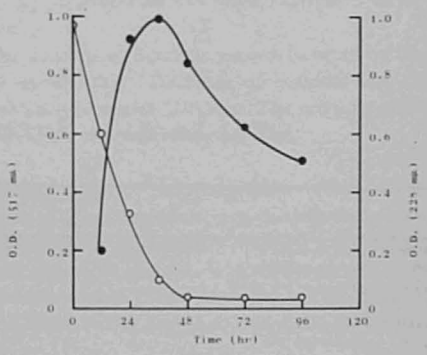


Fig. 2 Production of Myrosinase and Decrease of Sinigrin.

The bacterium was pre-cultured at 28°C on 500 ml of medium containing 1% meat extract, 1% yeast extract, 0.1% KH_2PO_4 and 0.1% $(NH_4)_2SO_4$, pH 7.0. After incubation for 16 hr, cells were harvested and washed twice with water and suspended in 20 ml of sterilized water. About 1 ml of suspension was inoculated in each 50 ml of synthetic medium containing 0.2% KH_2PO_4, 0.1% NH_4Cl, 0.1% $MgSO_4 \cdot 7aq$, 0.1% NaCl and 0.1% sinigrin. Enzyme activity was measured by the liberation of glucose. Decrease of sinigrin was measured by the absorbance at 228 mμ.

● ● Myrosinase activity
○ ○ Decrease of sinigrin

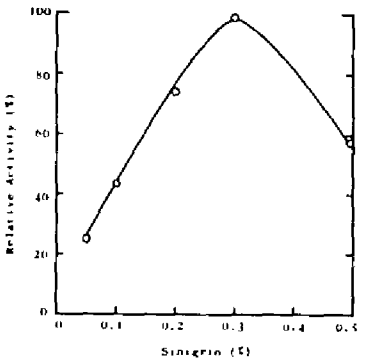


Fig. 3 Effect of Sinigrin Concentration on Enzyme Production.

The bacterium was pre-cultured as described in Fig. 2. After incubation for 16 hr, cells were harvested and washed twice with water and suspended in 20 ml of sterilized water. About 1 ml of suspension was inoculated in each 50 ml of synthetic medium containing 0.2% KH_2PO_4, 0.1% NH_4Cl, 0.1% $MgSO_4 \cdot 7aq$, and NaCl, in which the concentration of sinigrin was changed.

Table 3 Production of Acid and Gas from Carbon-Carbohydrate

	Acid	Gas		Acid	Gas
Glucose	+	+	Xylose	+	+
Galactose	+	+	Maltose	+	+
Fructose	+	+	Lafinose	+	+
Mannose	+	+	Dextrin	-	-
Treharose	+	+	Arabinose	+	+
Starch	-	-	Inulin	+	-
Lactose	+	+	Glycogen	-	-
Sucrose	+	+	Glycerol	+	-
Dulsitol	-	-	Mannitol	+	+
Adnitol	-	-	Inositol	+	-

Concentration of inducer

Bacterial myrosinase is also an induced enzyme that is produced by coexistence with the inducer such as sinigrin or mustard extract.

The effect of sinigrin concentration on best enzyme production was observed with about 0.3% of sinigrin (Fig. 3). However, it is not appropriate to use sinigrin as an inducing substance on a large scale, because it is rather on expensive chemical. Thus the mustard ext., which had a satisfactory effect on the myrosinase production by *Asp. niger*[2] and *Asp. sydowi*,[1] was tested for its ability to substitute for sinigrin. When it is used alone, the mustard ext. had a poor inducing ability on the myrosinase production. However, in the medium containing 0.01% sinigrin, the effect of adding of 6% mustard ext. showed the same enzyme production as in a medium containing 0.1% sinigrin (Fig. 4).

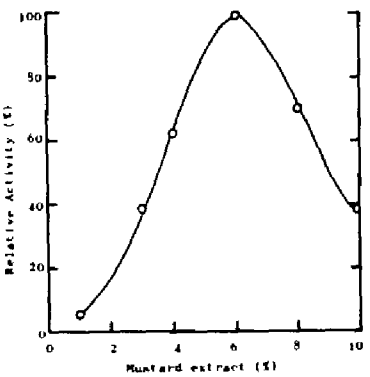


Fig. 4 Effect of Mustard Extract Concentration of Enzyme Production

The bacterium was pre-cultured as described in Fig. 2. After incubation for 16 hr, cells were harvested and washed twice with water and suspended in 20 ml of sterilized water. About 1 ml of suspension was inoculated in each 50 ml of synthetic medium containing 0.2% KH_2PO_4, 0.1% NH_4Cl, 0.1% $MgSO_4 \cdot 7aq$, 0.1% NaCl and 0.01% sinigrin, in which the concentration of mustard extract was changed.

Some substitutes for the inducer

It was investigated whether various glucosides and sugars were able to substitute for sinigrin on the myrosinase production. No enzyme was produced without the addition of inducer in the cases of *Asp. niger*[2] and *Asp. sydowi*.[1] The same was true for the bacterium. As shown in Table 4, no enzyme was produced without inducer.

Table 4 Effect of Some Substitutes for Sinigrin

Materials (0.3%)	Activity	Materials (0.3%)	Activity
Sinigrin	++	Glucose	-
Amygdalin	-	Galactose	-
Salicin	-	Xylose	-
Arbutin	-	Sucrose	-
α-Me-glucoside	-	Mannose	-
Cellobiose	-	Mannitol	-
Fructose	-	Sorbitol	-
Maltose	-		

As the nitrogen sources, ammonium sulfate, urea, thiourea, peptone and ammonium acetate were used instead of ammonium chloride. None of those had a remarkable effect on the myrosinase production.

The influence of yeast extract, some carbon-sources, glucose, glycerin and some organic acids were also investigated None showed positive effect.

Optimal conditions

In this study, the cultivation was carried out in a 2ℓ-shaking flask. To obtain the enzyme in a large scale, the cultivation by jar-fermentor was attempted several times. However, an expected myrosinase production was not obtained. Different varing compositions of the media and conditions were tried without success. Besides, the enzyme isolated by shaking flask cultivation, some times showed low activity, but, which could be recovered by maintaining the cultivation temperature at 28°C. From the foregoing considerations, optimal condition for the myrosinase production were ascertained and are summerized in Table 5.

Table 5 The Optimal Condition of Cultivation

KH_2PO_4	0.2 %
NH_4Cl	0.1
NaCl	0.1
$MgSO_4.7aq$	0.1
Sinigrin	0.01
Mustard extract	6.0
Initial pH	7.0
Temperature	28°C

in a 2L-shaking flask for 36-42 hr.

Chapter 2

Purification and General Characteristics

RESULTS

The procedure for the purification of bacterial myrosinase is shown in Fig. 1. The crude preparation, precipitayed at 0.3 to 0.8 ammonium sulfate saturation was dialyzed against 0.01 M potassium phosphate buffer, pH 6.3 and subjected to CM-Sephadex C-50 column (2ℓ, 8 x 40) chromatography. The absorbed protein was eluated with a linear gradient of 0.1 to 0.3 M sodium chloride in the same buffer. The active fractions were pooled. After concentration by 0.8 satn. of ammonium sulfate, gel-filtration on Sephadex G-200 column (5 x 80cm) were carried out. The eluated active fractions were pooled and concentrated by 0.8 satn. of ammonium sulfate. The precipitate obtained by centrifugation was dissolved in a small volume of same buffer and subjected to Sephadex G-100 column (2.6 x 90 cm). Finally, the enzyme was purified by rechromatography on Sephadex G-100 column (2.6 x 90cm). As shown in Fig. 2 a chromatographycally homogenous pattern of the enzyme was obtained. This filtrate was used throughout the following experiments as the purified enzyme preparation. A summary of purification of the enzyme is shown in Table 1.

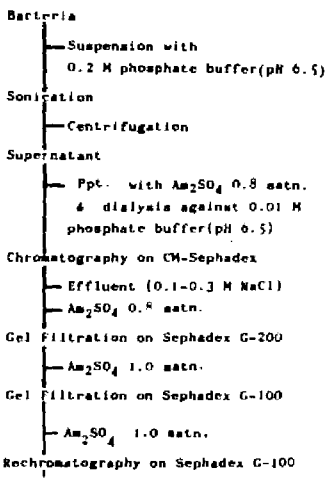


Fig. 1 Purification Procedure of Bacterial Myrosinase.

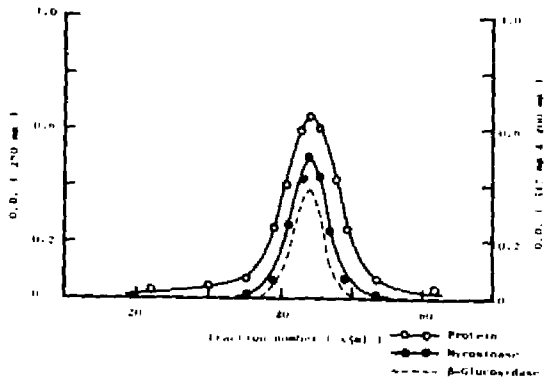
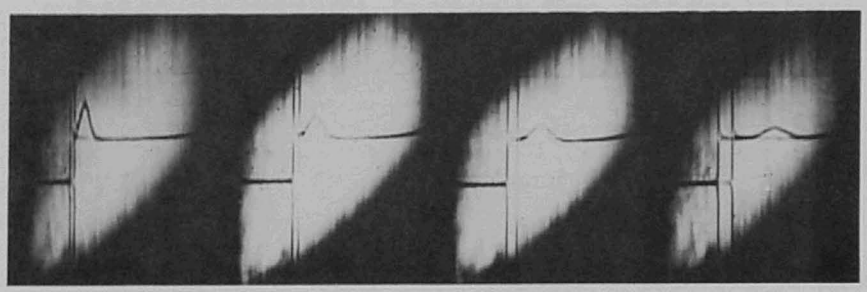


Fig. 2 Rechromatogram of Bacterial Myrosinase on Sephadex G-100.

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

Homogeneity of the bacterial myrosinase

The analysis of the purified enzyme by ultracentrifugation showed a single and symmetrical pattern (Fig. 3). The sedimentation coefficient, S_w^{20}, was caluculated to be 4.5 S from sedimentation velocity at 52,000 r.p.m..



10 min 24 min 36 min 60 min

Fig. 3 The Sedimentation Pattern of the Purified Enzyme.

Estimation of the molecular weight

The determination of the molecular weight was performed 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 the 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 temperature below 40°C.

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

Table I Summary of 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

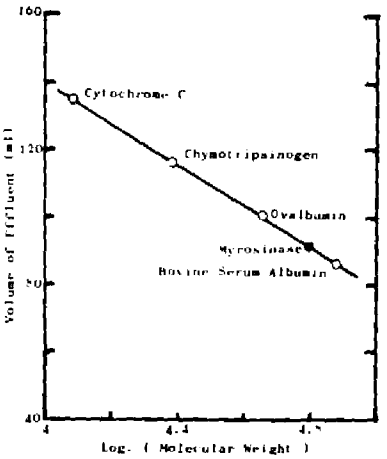


Fig. 4 Determination of Molecular Weight of Bacterial Myrosinase by Gel-Filtration on Sephadex G-100.

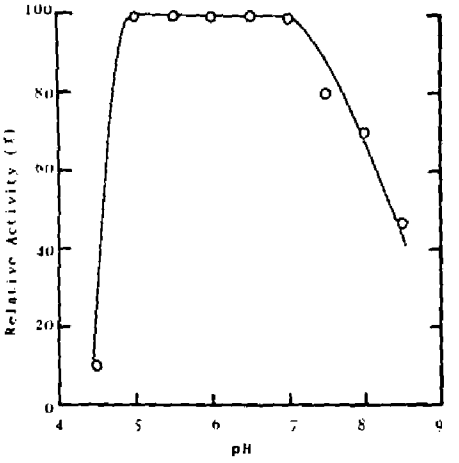


Fig. 6 pH-Stability Curve of Bacterial Myrosinase

The sample solution was preincubated at 20°C for 24 hr.

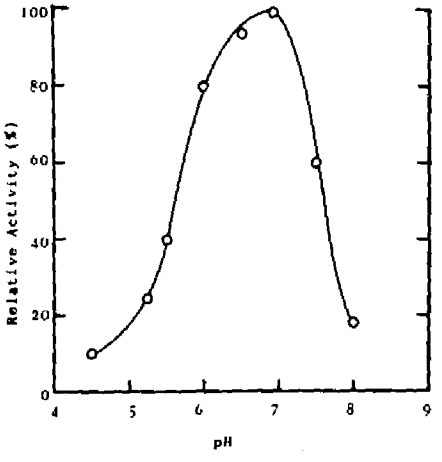


Fig. 5 pH-Activity Curve of Bacterial Myrosinase.

The enzyme activity was measured by the liberation of sulfate.

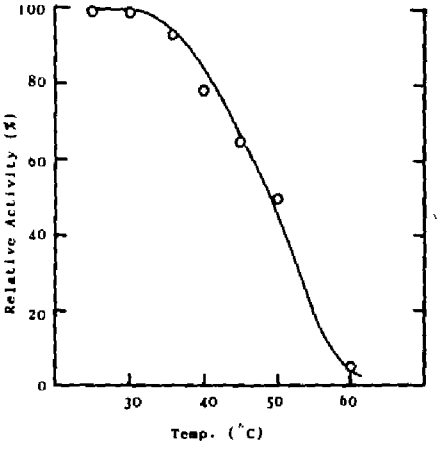


Fig. 7 Heat-Stability Curve of Bacterial Myrosinase.

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.

Effect of Various reagents

Sulfhydryl reagents, such as PCMB and iodoacetate showed no inhibitry effect. The activity was considerably inhibited by the chelating agent, EDTA, but not inhibited by o-phenanthlorine. δ-Gluconolactone, which is a specific inhibitor

Table 2 Effect of Various Reagents on Bacterial Myrosinase.

The enzyme solutions were preincubated with each inhibitor for 30 min at 37°C.

Reagents	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
O-Phenanthroline	10^{-3}	92
Dithiothreitol	10^{-4}	97
Mercaptoethanol	10^{-3}	130
δ-Gluconolactone	10^{-2}	65
Aascorbic Acid	10^{-4}	12

Table 3 Effect of Inorganic Salts on Bacterial Myrosinase.

All inorganic salts were preincubated for 30 min at 37°C.

	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}	42
$LiCl$	10^{-3}	120
$SnCl_2$	10^{-3}	103
$MnCl_2$	10^{-3}	93
$NaCl$	10^{-3}	95

on β-glucosidase, showed an inhibitory effect. L-ascorbic acid, which is the specific activator on plant myrosinase, showed a remarkably inhibitory effect on this enzyme (Table 2).

Many metal ions inhibited the enzyme activity to some extent. Paticularly, Cu^{+}, Cu^{++}, Hg^{++} and Fe^{++} inhibited the activity strongly (Table 3).

Table 4 Effect of Various Glucosides on Bacterial Myrosinase.

Enzyme activity was measured by the liberation of sulfate.

Glucoside(0.1M)	Relative Activity (%)
None	100
Salicin	23
Amygdalin	13
Arbutin	4
α-Me-glucoside	87
β-Me-glucoside	59
β-Phenylglucoside	34
p-Nitro-phenylglucoside	12

Table 5 Effect of Various Sugars on Bacterial Myrosinase.

Enzyme activity was measured by the liberation of sulfate.

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

Table 6 Substrate Specificity of Bacterial Myrosinase.

Enzyme activity was measured by the liberation of glucose. p-NPG hydrolysis was performed ad described in the text.

Substrate (4mM)	μmoles/min/ml
Sinigrin	1.073
Salicin	0
Arbutin	0
α-Me-glucoside	0
β-Me-glucoside	0
p-Nitro-phe-glucoside	0.616
β-phe-glucoside	0

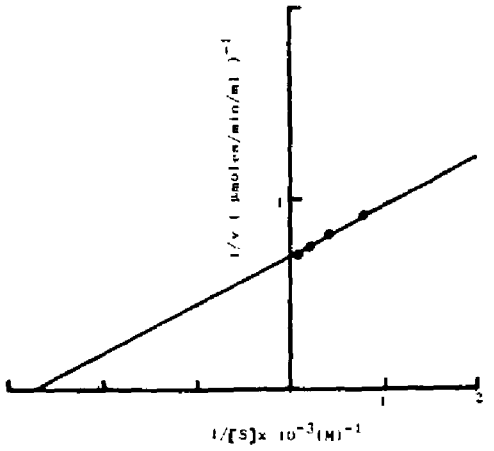


Fig. 8 Reciprocal Plot of Sinigrin Hydrolysis.

Enzyme activity was measured by the liberation of sulfate.

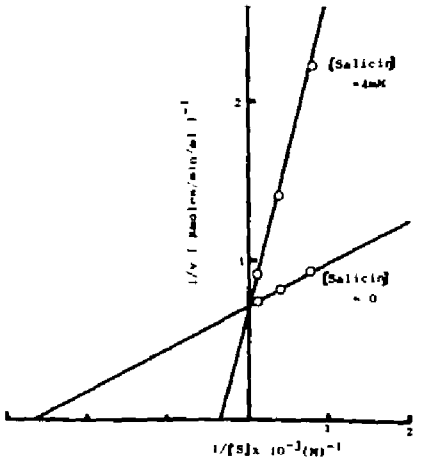


Fig. 10 Inhibitory Effect of Salicin on Sinigrin Hydrolysis.

Enzyme activity was measured by the liberation of sulfate.

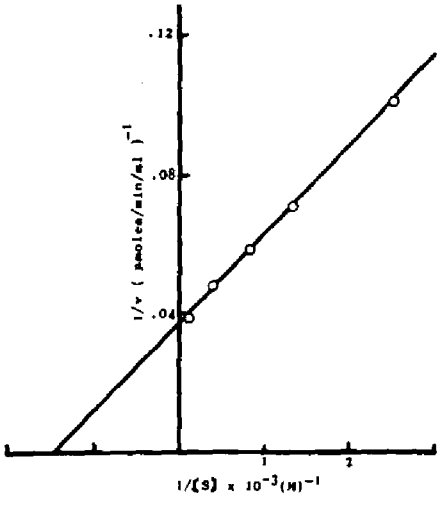


Fig. 9 Reciprocal Plot of p-NPG Hydrolysis.

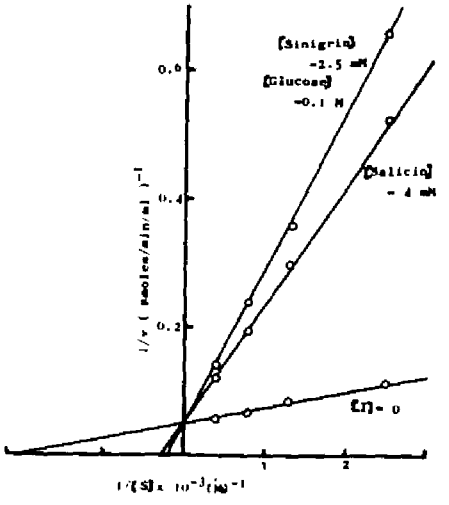


Fig. 11 Inhibitory Effect of Sugar and Glucosides on p-NPG Hydrolysis.

Effect of various glucosides and sugars

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

myrosinase, effect of glucosides and sugars was tested. All glucosides tested showed an inhibitory effect (Table 4). Paticularly, salcin, amygdalin, arbutin and p-NPG showed strong inhibitory effect. The effect of various sugars are shown in Table 5. All sugars tested showed inhibitory effect.

The inhibitory effect of salicin on sinigrin hydrolysis is demonstrated as Lineweaver-Burk plot in Fig. 10, plotted in the absence and presence of salicin. The inhibition by salicin is competitive. The Ki value for salicin was calculated to be 6.6×10^{-4} M.

Substrate specificity

The hydrolytic activity on various glucosides was tested (Table 6). The bacterial myrosinase hydrolyzed p-NPG besides sinigrin.

Effect of sugar and glucosides on p-NPG hydrolysis

The effect of sugar and glucosides was studied. As shown in Fig. 1 1, glucose, sinigrin and salicin showed competitive inhibition. The Ki 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 the bacterial myrosinase produced by *Enterobacter cloacae* no. 506 were investigated. Some properties of bacterial, fungous and plant myrosinases were compared (Table 7). On the effect of pH and heat, it is shown that the bacterial enzyme is rather stable like the plant enzyme and the fungous enzymes from *Aspergillus sydowi*. However, PCMB which

Table 7 Comparison of Some Properties of Myrosinases from Various Origins

	Plant	Asp. niger	Asp. sydowi	Enterobacter cloacae
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^{++} Cu^{++} Mn^{++}	Co^{++}	none
Inhibition	Cu^{++} Hg^{++}	Sn^{++} Hg^{++}	Fe^{++} Hg^{++}	Cu^{++} Hg^{++}
Molecular Weight	120,000 - 150,000	90,000	120,000	61,000
Km for Sinigrin	1.8×10^{-4} M	3.3×10^{-3} M	3.6×10^{-3} M	3.7×10^{-4} M
Km for p-NPG	2.0×10^{-3} M	1.5×10^{-3} M	1.0×10^{-4} M	7.1×10^{-4} M
Ki for Sinigrin (p-NPG hydrolysis)	2.0×10^{-4} M	3.8×10^{-3} M	3.8×10^{-3} M	3.0×10^{-4} M
Ki for Salicin (Sinigrin hydrolysis)	1.8×10^{-1} M		1.6×10^{-1} M	6.6×10^{-4} M

inhibited the fungous and plant myrosinase showed no inhibitory effect on the bacterial myrosinase. EDTA which showed no inhibitory on the plant and fungous myrosinase inhibited the bacterial one and metal ion, Hg^{++}, inhibited all the enzymes. L-Ascorbic acid which is the specific activator on the plant enzyme inhibited the bacterial enzyme. Plant myrosinase possesses a rather large moleculer weight of 150,000, while, bacterial enzyme had the smallest weight in the four myrosinases.

As in the 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 on which the hydrolysis of sinigrin. While, on the Km values for sinigrin and p-NPG, the bacterial myrosinase has a different tendency from the fungous myrosinase, because the ratio of Km values for sinigrin to p-NPG is in the order plant<bacterial<*Asp. niger*<*Asp. sydowi*. These facts suggest that the bacterial myrosinase may considered to be a β-glucosidase which has high affinity for mustard oil glucoside. Pigman,[9] Nagashima and Uchiyama,[10, 11] and Tsuruo and Hata[12] 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, but the bacterial myrosinase is located between plant and fungous enzyme and is thought to be rather similar to the plant enzyme than the fungous enzymes. 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 presense of inducer such as sinigrin and mustard extract.

The differences in behaviours and characteristics of the four enzymes becomes an interesting factor. Particularly, it is noteworthy that ascorbic acid has a very prominent effect on each enzyme.

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SUMMARY AND CONCLUSION

PART I

In Chapter 1:

Four proteins (F-IA, B, F-IIA & F-IIB), having myrosinase activity, were separated and purified from mustard powder. Each enzyme was shown to be homogeneous chromatographically, ultracentrifugally and Disc electrophoretically. Molecular weights obtained by gel-filtration and sedimentation equilibrium were 153,000 (F-IA, F-I B & F-IIA) and 125,000 (F-IIB). Sedimentation coefficients were 6.8 S (F-IA, B & F-IIA) and 5.8 S (F-IIB). Stokes radius (Å), diffusion coefficient (cm^2/sec) and frictional ratio (f/fo) were 47, 4.28×10^{-7} and 1.33 (F-IA, B & F-II1), and 43, 4.67×10^{-7} and 1.29 (F-IIB), respectively. Isoelectric points were pH 4.6 (F-IA, B & F-IIA) and pH 4.8 (F-IIA). The enzymes were glycoprotein with 9~22% carbohydrate. Amino acid composition of F-IA, B and F-IIA were very similar, but in case of F-IIB, glutamic acid, arginine and methionine contents were higher and aspartic acid and histidine contents were lower than others. The molecular weights estimated from SDS-polyacrylamide gel electrophoresis were 40,000 (F-IA, B & F-IIA) and 30,000 (F-IIB), respectively, and hence the enzymes are considered to have at least 4 subunits. From these results, it may be confirmed that F-IA, B & F-IIA have striking resemblances and only F-IIB is rather different.

In Chapter 2:

The functional groups of the plant myrosinase was investigated using reagents which discriminate the states of amino acids in protein. The enzymatic activity was inhibited by fluorodinitrobenzene (FDNB), trinitrobenzenesulfonic acid (TNBS) and monochlorotrifluoro-*p*-benzoquinone (CFQ), which are specific reagents for amino group, *p*-mercuribenzoate (PCMB) and 5-5′-dithio-bis-(2-nitrobenzoic acid), specific for -SH group, and *p*-diazobenzenesulfonic acid, specific for imidazole group. The rates of inhibition by FDNB and TNBS were accelerated by the addition of ascorbic acid (10^{-3} M). The enzyme was inactivated when five amino groups of the enzyme reacted with CFQ. The enzyme was also inactivated when two -SH groups reacted with Ellman reagent, although four -SH groups were found by Ellman reagent in the denatured enzyme by SDS.

Plant myrosinase was found to be quite sensitive to photooxidation catalyzed by methylene blue. Amino acid analysis indicated that loss of the activity was due to degradation of histidyl residue of the enzyme. These results suggest that amino group, -SH group and histidyl residue constitute the active sites of the enzyme.

In Chapters 3 and 4:

The activation of myrosinase was observed above 5×10^{-5} M ASA concentration, and the activation maximum was around 10^{-3} M ASA.

The presence of dissociation and association of the enzyme by ASA was investigated by gel-filtration and ultra-centrifugal analysis. The enzyme was neither dissociated nor associated by ASA.

The procedure for quantitation of ligand binding to proteins by dialysis rate measurements has been applied to binding of L-ascorbic acid by myrosinase. Myrosinase has 4 sites per molecule which bind ASA rather strongly ($Kd = 0.1 \times 10^{-4}$ M), and at least one additional site which binds ASA less strongly ($Kd = 0.9 \times 10^{-4}$ M). Myrosinase activity is most strongly activated when four ASA molecules are bound to the enzyme, but it is inhibited when five ASA molecules are bound.

Ascorbic acid analogues could not activate the enzyme.

It was found that the enzyme protein is comformationally changed by the addition of ASA in the photometrical analysis. Approximately, 1.5 amino residues appeared on the surface of the enzyme and about 2.3 tryptophanyl residues are buried in the molecule when ASA is added (1 mM).

Myrosinase and β-glucosidase activities were strongly inhibited by p-mercurybenzoate (PCMB) (0.1 mM) with or without ASA. Diethylpyrocarbonate (DEP) inhibited the myrosinase activity than β-glucosidase. 2-Methoxy-5-nitrotropone (MNT) inhibited ASA treated myrosinase when the activity was measured without ASA, but the activity was not affected when the activity was determined with ASA. This phenomenon was not prominent in β-glucosidase activity.

Optimum-temperature for the myrosinase activity was at about 55°C without ASA, but with ASA was at about 35°C. β-Glucosidase activity was the same (55°C) with or without ASA.

A schematic model interpreting at the interaction of ASA with the enzyme and the active center of the enzyme is proposed.

I concluded that the activation of the plant myrosinase is occured by the alteration of the enzyme's protein structure caused by the binding of ASA exerted no effect on the area which adsorbed glycon moiety in the substrate site, and by neither the oxidation-reduction reaction of ASA nor the association-dissociation of the enzyme. I considered that sulfhydryl groups are essential to the catalytic action of the plant myrosinase and amino and histidyl residues are situated on the region which is altered by the addition of ASA, that is, it is close to aglycon moiety and the locations of them are changed to accelarated the enzyme action by the binding of ASA to the effector site.

PART II

In Chapter 1:

In order to obtain fungous myrosinase, *Aspergillus sydowi* IFO 4284 was cultured on a medium containing mustard seed extract for 2 weeks. Myrosinase in the broth was purified about 150 fold by precipitation with ammonium sulfate and chromatography on DEAE-cellulose and DEAE-Sephadex. Comparison of thioglucosidase and sulfatase activities of the myrosinase preparation using pH-activity, pH-stability and temperature-stability curves revealed no differences from each other. The chromatograms of the two activities on DEAE-Sephadex showed good agreement. Consequently, the myrosinase produced by *Aspergillus sydowi* was concluded to be a single β-thioglucosidase, not a mixture of thioglucosidase and sulfatase.

In Chapter 2:

The effects of various reagents on *Aspergillus sydowi* myrosinase were studied.

The enzymatic activity was stimulated by cobalt (II), zinc (II) and magnesium ions and inhibited by mercury (II), iron (II) and copper (II) ions. However, metal-complexing agents, SH reagents and diisopropylfluorophosphate showed no effects on enzymatic activity. In contrast to plant myrosinase, this enzyme was neither activated nor inhibited by any concentrations of L-ascorbate. Glucose and salicin were competitive inhibitors for the enzyme. High concentrations of sodium chloride inhibited the enzyme.

From the inhibition modes of sugars and β-glucosides and from that of sodium chloride against the enzyme, a similarity of the enzyme to β-glucosidases was shown.

In Chapter 3:

β-Glucosidase activity of fungous myrosinase was confirmed using *p*-nitrophenyl β-glucoside as a substrate. This activity was revealed to be due to the myrosinase itself. Experimental results indicated a resemblance of fungous myrosinase to β-glucosidases similar to plant myrosinase. The relationship between fungous and plant myrosinases to the β-glucosidases are discussed from the view of the substrate specificity of these enzymes. The conclusions are that distinction between plant and fungous myrosinases and the β-glucosidases are not as strict as previously thought, and the myrosinases should be considered β-glucosidases highly specialized for the hydrolysis of mustard oil glucoside.

In Chapters 4 and 5:

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).

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 *Km* 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.

PART III

In Chapters 1 and 2:

Screening test for obtaining microorganisms which produce myrosinase activity was carried out. One strain of microorganism showed strong ability to produce myrosinase activity.

The morphological and physiological characteristics of this strain were studied.

The organism was identified as an *Enterobacter cloacae* no 506.

Enzyme production was induced by the addition of 0.01% sinigrin and 6% mustard extract to the culture medium. When the strain was cultivated at 28°C in a medium containing 0.01% sinigrin, 6% mustard ext., 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.1% NaCl and 0.1% $MgSO_4 \cdot 7aq$, (pH 7.0), the highest activity was obtained after 36 ~ 40 hr cultivation.

Myrosinase in the 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 chromatographically and ultracentrifugally. Molecular weight obtained by gel-filtration was 61,000 and sedimentation coefficient was 4.5 S. Maximum activity occurred at pH 6.8, and the enzyme was stable, in a pH range of 5.0 to 7.0 below 40°C for 24 hrs. Copper (I), (II), mercury (II) and ferrous (II) ions inhibited the activity strongly. 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. The comparison of some properties of bacterial, fungous and plant myrosinases are discussed.

The above experimental results indicate that the striking family relation was observed between the myrosinases and the β-glucosidases. And I concluded that the myrosinases are a kind of β-glucosidases highly specialized for hydrolysis of mustard oil glucosides.

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