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Purification and Characterization of Peroxidase Isozyme C from Mung Bean

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녹두의 Peroxidase Isozyme C의 생화학적 성상

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Abstract

Peroxidase isozyme C was isolated from mung bean cotyledon and purified to homogeneity as ascertained by chromatography and polyacrylamide gel electrophoresis, and then crystallized. Purification procedures included ammonium sulfate precipitation and column chromatography on Sephadex G-75, DEAE-cellulose and DEAE-Sephadex A-50. Peroxidase isozyme C was purified about 63 fold with 5% recovery. Isozyme C showed optimal activity at pH 5.0 with o-dianisidine and at pH 6.0 with guaiacol as substrate, and the optimal temperature was 70°C. Molecular weight of 50,000 was estimated for the isozyme C by SDS-polyacrylamide gel electrophoresis. At 70°C, it took 30 min to inactivate the isozyme to 50%, and at 80°C, this isozyme was almost completely inactivated in 20 min. The Km value of isozyme C for o-dianisidine was 0.11mM and that for guaiacol was 60.98mM using hydrogen peroxide as cosubstrate, and the kinetic pattern showed a competitive cyanide inhibition with respect to substrate. The crystalline structure of isozyme C was rectangular in shape.

Introduction

Peroxidase (Oxidoreductase: E.C. 1.11.1.7) is a heme protein commonly found in higher organisms.[1] This enzyme is often found in multiple molecular forms (isozymes) which are recognized by their distinct electrophoretic mobilities. Multiple forms of peroxidase have been found to vary with different organs in maize[2], peas[3,4], and barley[5]. Hoyle[6], by isoelectric focusing, has demonstrated that up to 42 isoper oxidases could occur in horseradish roots. In tomato plants, Evans and Aldridge[7] separated out 6 isoperoxidases and Kokkinakis et al.[8] iso lated an isoperoxidase from tomato pericarp tissue to 85% purity and studied its kinetic properties. In later reports, Lee et al.[9] reported 5 isoperoxidases from mung bean roots and partially purified two of the isozymes. While all peroxidase isozymes appear to catalyze the same reaction, the individual isozymes may markedly differ in physicochemical and kinetic properties.[10]

In order to determine the biological characteristics of the peroxidases, it is necessary to

isolate with purity and study them individually. This paper describes the isolation and purification of the major peroxidase isozyme from mung bean cotyledon.

Materials and Methods

Materials

O-dianisidine dihydrochloride, guaiacol, tris (hydroxy-methyl) aminomethane, bovine serum albumin, 2-mercaptoethanol, marker proteins for molecular weight determination were purchased from Sigma. Sephadex G-75, DEAE-cellulose, DEAE-Sephadex A-50 were obtained from Pharmacia. Electrophoresis reagents were obtained from Bio-Rad. All other reagents were used of analytical grade.

Germination of mung bean

Mung bean (*Phaseolus aureus* Roxb.) seeds were germinated for 3 days in a growth chamber in the dark at 27°C with the application of tap-water 4 times a day after imbibition for 12 hrs.

Enzyme extraction and assay

Cotyledon germinated for 3 days was ground in a mortar with 3 volumes(w/v) of phosphate buffer(pH 6.0) and centrifuged at 10,000×g for 10 min. The supernatant was collected and referred to as crude homogenate.

The activity of peroxidase was assayed by the use of two substrates (o-dianisidine and guaiacol). The enzyme activity was measured by the procedure described in the Worthington enzyme manual[11] (with o-dianisidine) and by the method of Pütter[12] (with guaiacol). One unit of the enzyme activity was defined as the amount of enzyme decomposing 1μmole of peroxide per min at 30°C, and the specific activity expressed as unit of enzyme activity per mg protein. The amount of protein was determined by the method of Lowry et al.[13] with crystallized bovine serum albumin as standard.

Purification of peroxidase

The crude homogenate of cotyledon germinated for 3 days was purified by the modified procedures of Kokkinakis et al.,[8] Shannon et al.[14] and Claiborne et al.[15]

The crude homogenate was subjected to fractional precipitation with saturation of ammonium sulfate from 30 to 80%. The precipitate was dispersed in 20mM Tris-HCl buffer (pH 8.2) and dialyzed against the same buffer. The fraction was transferred to Sephadex G-75 (2.0 ×43.0 cm) equilibrated with Tris-HCl buffer (pH 8.2), and the column washed with the same buffer. The eluates (3ml/tube) were monitored for protein absorbance at 280nm by a spectrophotometer (Pye Unicam PU 8800, Philips) and also assayed for peroxidase activity. The eluates containing high enzyme activity were collected and applied to a column of DEAE-cellulose (2.0×40.0cm) which was pre-equilibrated with 20mM Tris-HCl buffer (pH 8.2). Then the column was eluted by a linear gradient with NaCl (from 0 to 1.0M) in the same buffer. The fractions pooled and concentrated were dialyzed for 12 hrs against Tris buffer, pH 8.2. The dialyzed enzyme was loaded on a DEAE-Sephadex A-50 column (2.0×40cm) pre-equilibrated with 20mM Tris-HCl buffer, pH 8.2. The column was washed with 200 ml of the same buffer and eluted by a linear gradient of 0~1.0M NaCl in the buffer at 36ml/hr, and 3 ml fractions were collected from the time the column was charged. The high enzyme fractions were collected and lyophilized. All the procedure involved in column chromatography were carried out at 4°C.

Disc gel electrophoresis

Electrophoresis in polyacrylamide gel at pH 8.3 was performed as described by Davis[16] using 6% polyacrylamide gel. Peroxidase isozyme was identified by the procedure of Lee et al.[9] Ten percent polyacrylamide gel containing sodium dodecyl sulfate was run according to the

procedures by Weber et al.[17] Electrophoresis was run at 8mA per tube until the bromophenol blue tracking dye reached the bottom of the gel. As the standard marker proteins (in daltons), bovine albumin (66,000), ovalbumin (45,000), pepsin (34,700), trypsinogen (24,000), lactoglobulin (18,400) and lysozyme (14,300) were used adopted.

Amino acid composition and crystallization

The amino acid composition of the peroxidase was determined by the method of Spackman et al.[18] with a L.K.B amino acid analyzer. Crystallization was performed according to the methods of Morita et al.[19] Enzyme powder was dissolved in minimum of water and saturated ammonium sulfate solution was added dropwise at 15°C until a faint turbidity appeared (ca 35 %), and then the mixture chilled to 0°C. The solution which has become clear again, was left stood at 4°C. The crystallization of peroxidase was complete in 48 hrs.

Results and Discussion

Purification of peroxidase

Table 1 summarizes the results of purification procedures for mung bean peroxidase. The specific activity of peroxidase in crude extract was about 0.6 units per mg protein. The bulk of peroxidase was eluted from the DEAE-cellulose column with a NaCl gradient between 0.35 and 0.55M. This chromatography of the peroxidase revealed two major peak (Fig. 1). In this paper, the first peak was named peroxidase isozyme C which was more purified and investigated for its characterization and kinetic behavior. The second peak designated isozyme D will be discussed elsewhere[20] on its characterization and kinetic behaviors. This step resulted in 16% recovery of the enzyme with 14 fold purification. The final preparation of the peroxidase by DEAE-Sephadex A-50 was purified about 63 fold with 5% recovery in 0.35M of NaCl (Fig. 2).

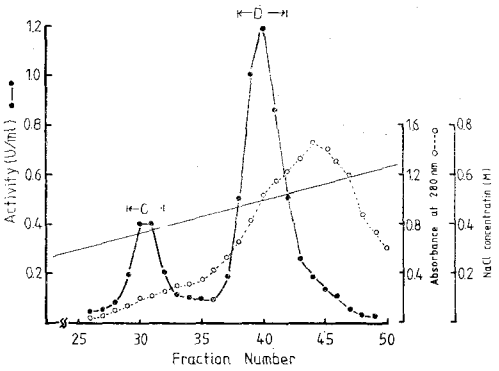

Fig. 1. Elution profile of peroxidase from a DEAE-cellulose column (2.0×40.0cm) equilibrated with 20mM Tris-HCl buffer, pH 8.2. The enzyme was eluted with a linear gradient of NaCl at flow rate of 45ml/hr and fractionated 3 ml. Protein was estimated by absorbance at 280nm (○···○), and enzyme activity was expressed as the unit per ml (●—●).

Table 1. Summary of purification of peroxidase isozyme C from cotyledon of mung bean

Procedure	Total protein (mg)	Total activity (U)	Total activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	352.8	223.0	0.6	1.0	100.0
30~80% $(NH_4)_2SO_4$	118.9	173.6	1.5	2.3	77.9
Sephadex G-75	19.3	138.7	7.2	4.9	62.2
DEAE-cellulose	2.6	35.0	13.5	21.3	15.7
DEAE-Sephadex A-50	0.3	10.6	39.9	63.1	4.7

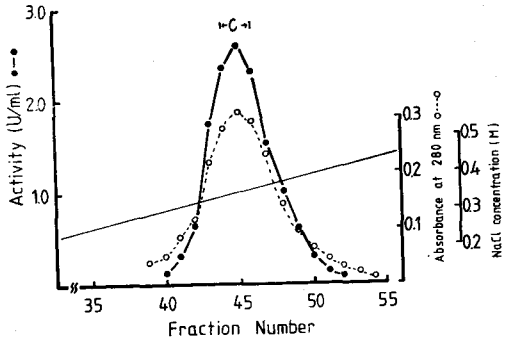

Fig. 2. Elution profile of peroxidase isozyme C from a DEAE-Sephadex A-50 column (2.0×40.0cm) equilibrated with 20mM Tris-HCl buffer, pH 8.2. The enzyme was eluted with a linear gradient of NaCl at flow rate of 36ml/hr and fractionated 3ml. Protein was estimated by absorbance at 280 nm (○···○), and enzyme activity was expressed as the unit per ml (●—●)

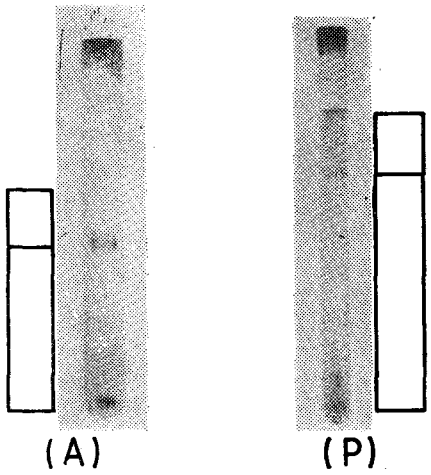

Fig. 3. Polyacrylamide disc gel electrophoresis of the purified peroxidase isozyme C. A: Activity staining, P: Protein staining

Criteria for purity and molecular weight

Purity of the peroxidase isozyme was confirmed by two pieces of evidence. First, chromatographic separation of isozyme C yielded a single protein peak and peroxidase activity. Second, isozyme C migrated as a single protein band with a characteristic electrophoretic mobility. The electrophoretic mobility of the protein band coincided with peroxidase activity, its Rm value being 0.24 (Fig. 3). On the basis of these tests it appeared that isozyme C was essentially free of impurities. The molecular weight of isozyme was estimated by SDS polyacrylamide gel electrophoresis. The isozyme C revealed only one protein band with a molecular weight of 50,000 dalton (Fig. 4), suggesting that this enzyme is composed of only one polypeptide. Molecular weight of isozyme C was relatively larger than those obtained with the enzymes from other sources such as soybean,[21] horseradish[22] and tomato.[23] The amino acid composition of the isozyme C is presented in Table 2. The predominance of acidic amino acid residues over basic amino acid residues in isozyme C consisted with the amino acid composition of several other peroxidases.[11,22]

General characteristics of peroxidase

The pH optimum of mung bean peroxidase with o-dianisidine as substrate was found to be 5.0 and with guaiacol to be 6.0. The pH optimum for isozyme C was about 1 pH unit lower

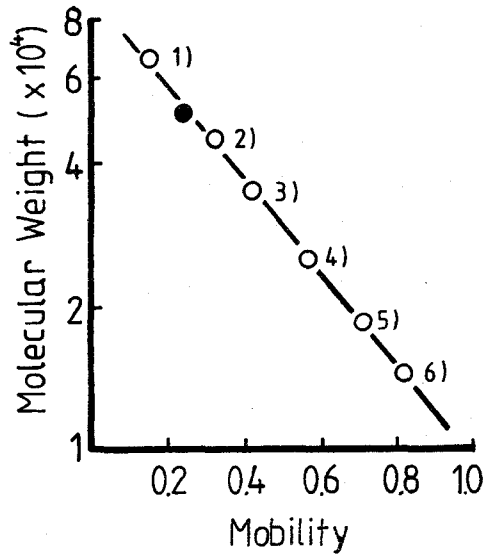

Fig. 4. Molecular weight determination of puri fied peroxidase isozyme C by SDS poly acrylamide gel electrophoresis.
1) Bovine albumin (66,000), 2) Ovalbumin (45,000), 3) Pepsin (34,700), 4) Trypsinogen (24,000), 5) Lactoglobulin (18,400), 6) Lysozyme (14,300)

Table 2. Amino acid composition of peroxidase isozyme C

	X	Y
Asp.	12.37	46.47
Thr.	4.82	20.23
Ser.	9.54	45.39
Glu.	10.13	34.42
Pro.	5.49	23.84
Gly.	7.28	48.48
Ala.	6.25	35.08
Cys.	4.11	8.55
Val.	6.03	25.74
Met.	1.29	4.32
Ile.	6.52	24.85
Leu.	5.28	20.03
Tyr.	2.44	6.73
Phe.	5.48	16.59
His.	1.56	5.03
Lys.	8.73	29.86
Arg.	3.95	11.34

X: g of amino acid residue per 100g protein
Y: Number of residues per protein molecular

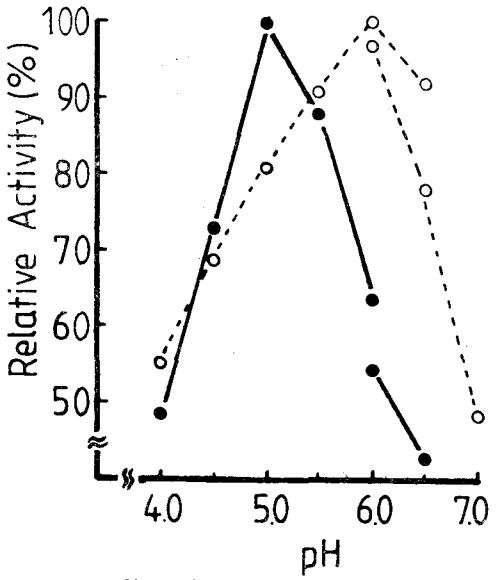

Fig. 5. Effect of pH on the activity of purified peroxidase isozyme C with o-dianisidine (●—●) and guaiacol (○···○) as substrate. Acetate buffer was used from pH 4.0 to 6.0 and phosphate buffer from pH 6.0 to 7.0. Activity at the optimal pH was expressed as 100%.

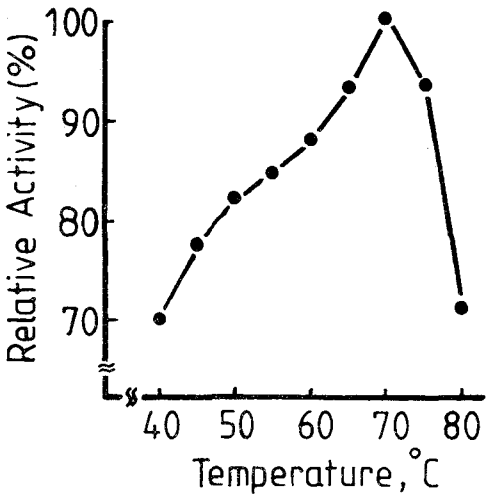

Fig. 6. Effect of incubation temperature on the activity of purified peroxidase isozyme C

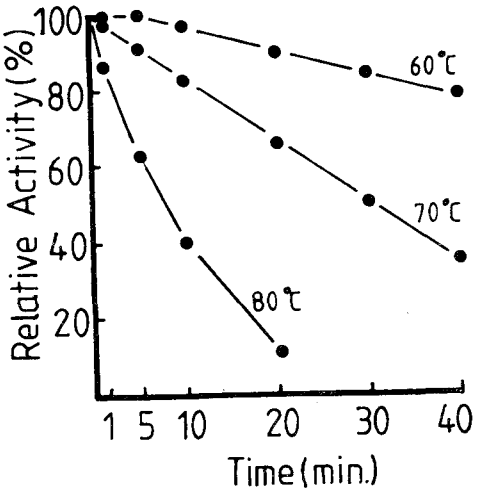

Fig. 7. Effect of heating on the activity of purified peroxidase isozyme C. Heated enzyme solutions were cooled in ice and then each enzyme solution were assayed at 30°C, pH 5.5.

with o-dianisidine than with guaiacol as substrate (Fig. 5). The optimum temperature of isozyme C tested between 30°C and 80°C was 70°C (Fig. 6). Plant peroxidases, in general, are very stable to heat.[24] The effect of heat on isozyme C was investigated at pH 5.5 under various temperatures (Fig. 7). When heated for 30 minutes at 70°C, the remaining activity

of isozyme C dropped nearly to 50%. After 20 minutes of heating at 80°C, the remaining activity of isozyme C dropped to 15%. This result shows that isozyme C is considerably stable to heat. Table 3 shows the effect of metal ions on isozyme C activity. In high concentration (5.0mM), all the ions except Hg^{++} employed were found to have remarkable activation effects on isozyme C. Particularly, Zn^{++}, Fe^{++} and Cu^{++} activated the isozyme C from about 1.7 to 2.4 times.

Kinetic properties and crystallization

Substrate concentration studies by Lineweaver-Burk plots[25] showed that apparent Km for o-dianisidine was 0.11mM and that for guaiacol was 60.98mM using hydrogen peroxide as cosubstrate (Fig. 8, 9). The apparent Km for hy-

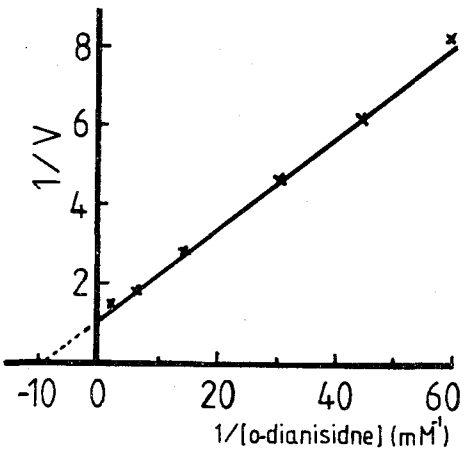

Fig. 8. Effect of substrate (o-dianisidine) concentration on the activity of purified peroxidase isozyme C as cosubstrate H_2O_2.

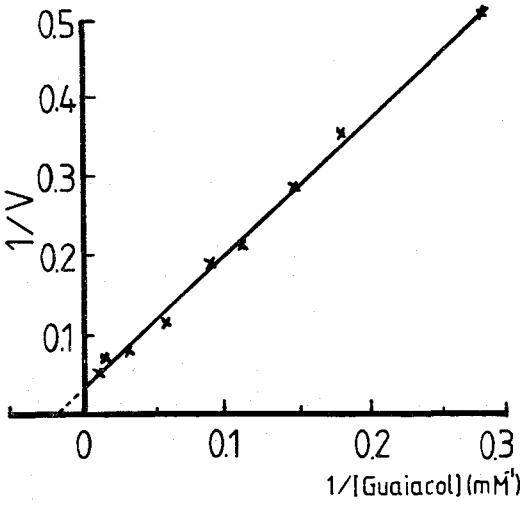

Fig. 9. Effect of substrate (guaiacol) concentration on the activity of purified peroxidase isozyme C as cosubstrate H_2O_2.

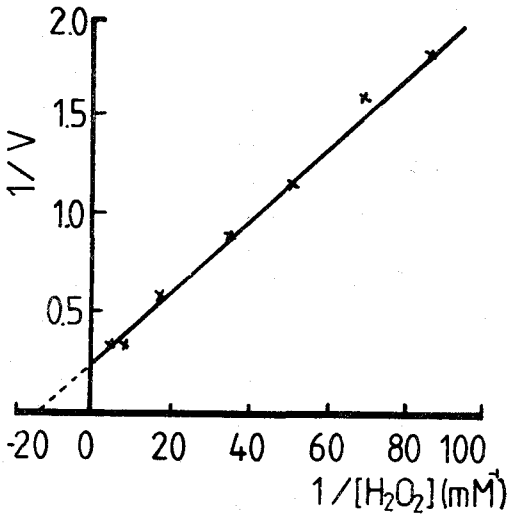

Fig. 10. Effect of substrate (H_2O_2) concentration on the activity of purified peroxidase isozyme C as cosubstrate o-dianisidine

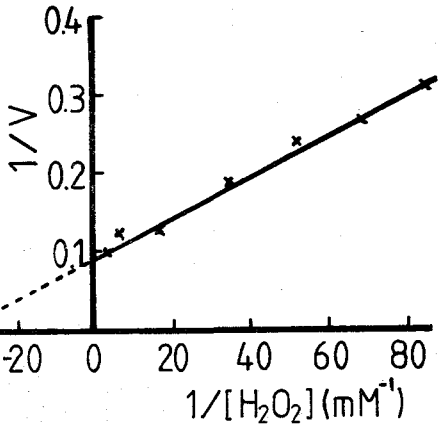

Fig. 11. Effect of substrate (H_2O_2) concentration on the activity of purified peroxidase isozyme C as cosubstrate guaiacol

Table 3. Effect of metal ions on peroxidase activities

	Cation conc.	Co^{++}	Zn^{++}	Ca^{++}	Mn^{++}	Mg^{++}	Fe^{++}	Cu^{++}	Hg^{++}	None
Isozyme C	0.5mM	101.9	102.8	93.3	98.3	91.9	118.0	115.9	8.8	100.0
	1.0mM	108.1	103.2	101.5	104.5	98.5	118.4	116.2	3.5	100.0
	5.0mM	135.6	176.2	121.9	109.8	110.3	236.9	170.6	—	100.0

* Enzyme activity of the control was taken as 100

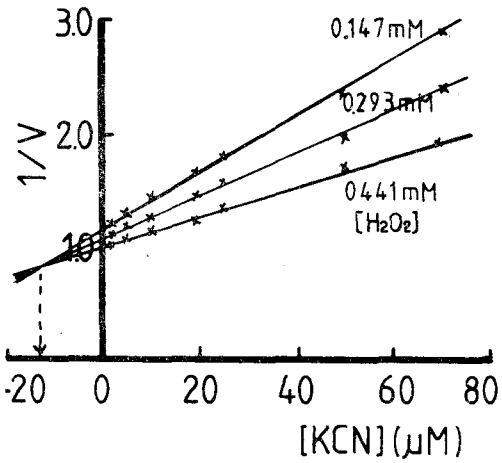

Fig. 12. Effects of cyanide on peroxidase isozyme C oxidation of o-dianisidine at pH 5.5 and three concentrations of H_2O_2

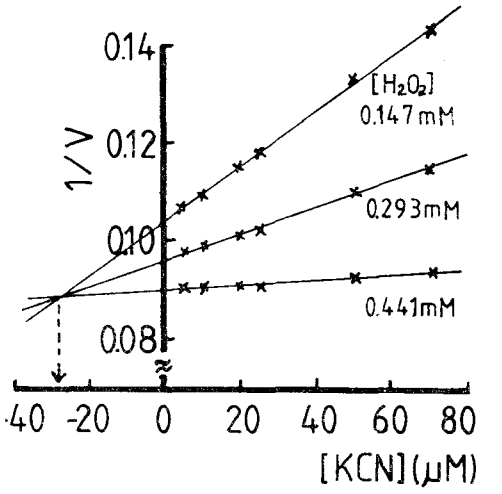

Fig. 13. Effects of cyanide on peroxidase isozyme C oxidation of guaiacol at pH 6.0 and three concentrations of H_2O_2

drogen peroxide using o-dianisidine and guaiacol as cosubstrate were 0.08mM and 0.03mM, respectively (Fig. 10, 11). The kinetic pattern depicted in Fig. 12, 13 by a Dixon plot[26] indicates competitive inhibition of peroxidase isozyme C by cyanide. The inhibition constant (Ki) of cyanide was determined to be 15μM with o-dianisidine and 31μM with guaiacol as substrate. Because of its higher Ki compared with those of peroxidase from other species[21,27] mung bean peroxidase was apparently less sensitive to inhibition with cyanide than horseradish and soybean peroxidase.

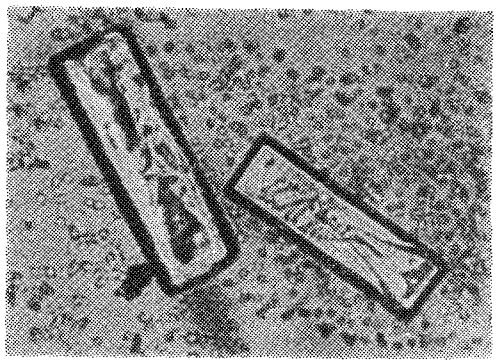

Fig. 14. Microphotograph of crystalline peroxidase isozyme C of mung bean cotyledon(×100)

Microphotograph of crystalline peroxidase isozyme C of mung bean is shown in Fig. 14. The crystalline form of isozyme C was rectangular in shape.

초 록

녹두의 자엽에서 peroxidase isozyme을 $(NH_4)_2SO_4$ 침전, Sephadex G-75, DEAE-cellulose 및 DEAE-Sephadex A-50 column chromatography

등으로 63배 정제하여 그 특성 및 결정구조를 조사하였다. Isozyme C는 Rm 값이 0.24로서 분자량이 50,000 dalton인 단량체였다. 이 효소의 반응 최적 pH는 o-dianisidine에 대하여 5.0, guaiacol에 대해서는 6.0, 반응 최적온도는 70°C였으며 열에 대해서도 비교적 안정하였다. o-dianisidine과 guaiacol에 대한 Km 값은 각각 0.11mM, 60.98mM이었으며, isozyme C에 대한 기질과 cyanide는 경쟁적 저해형식을 나타내었다. Isozyme C는 평면상 직사각형의 결정구조를 하고 있었다.

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