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Isolation and Characterization of Two Isoperoxidases from Mung Bean Seedling

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綠豆에서 Peroxidase 同位 酵素들의 分離와 酵素的 特性

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抄 錄

發芽初期 綠豆의 部位別 peroxidase 活性은 뿌리가 他部位보다 약 1.5~3.5배 더 높은 酵素活性을 나타내었으며 發芽가 進行됨에 따라 子葉, 뿌리에서는 酵素活性이 增加하다가 점차 減少하는 傾向을 보인 반면에 他部位에서는 계속 減少하는 傾向을 보였다. 電氣泳動上 isozyme의 수는 發芽, 生育이 進行됨에 따라 모든 部位에서 계속 增加하여 活性度와는 無關하게 發芽 후 6日째에 가장 많이 나타났다. Ammonium sulfate에 의한 蛋白質의 分劃, Sephadex G-75 Column, DEAE-cellulose column한 結果 isozyme A는 16배 精製되었으며, isozyme B는 조금도 純度가 增加되지 않았으며 isozyme A와 B의 電氣泳動上 Rm값은 各各 0.22와 0.62였다. Isozyme A와 B 모두 pH5.6에서 最大 活性을 나타내었으며 또한 反應最適溫度는 各各 65°C와 70°C였으며, 熱에 대해서도 比較的 安定한 結果를 얻었다. Lineweaver-Burk polt를 利用하여 Km값을 測定해 본 結果 0-dianisidine에 대한 isozyme A는 0.071mM, isozyme B는 0.052mM이었으며 H_2O_2에 대한 isozyme A는 0.28mM, isozyme B는 0.23mM이었다.

Introduction

Peroxidase (donor: H_2O_2, oxidoreductase: E.C. 1.11, 1.7) is a heme-containing glycoprotein, and is an enzyme defined by its ability to oxidize a wide variety of hydrogen donors such as phenolic substances, cytochrome c, nitrite, leuco-dyes, amines and indole in the presence of hydrogen peroxide.[1] In addition, peroxidase is capable of oxidizing several compounds in the absence of H_2O_2 e.g., chaleones, tyrosine, tyramine, $NADH_2$, $NADPH_2$, and thiols.[2~5]

Despite the fact that peroxidases are among the oldest enzymes known and are widely distributed in nature, very little is known about their function(s). However, peroxidase activity has been implicated in indole acetic acid oxidation,[6,7] ethylene biosynthesis,[8] hydroxylation of proline[9] and lignification,[10,11] and so is po-

tentially of considerable importance. Peroxidase is known to exist in plant tissues in multiple forms. Exposure of the plant tissue to factors such as wounding,[12] disease,[13] gamma irradiation,[14] day-light and temperature[15] often results in pronounced changes in the peroxidase isozyme pattern of the tissue.

The isozyme was first detected by Theorell[16] in horseradish roots. Isozymes of peroxidase were known to occur in a variety of tissues in a large number of plant species,[17~19] and were studied for enzymatic properties.[20~27] However, it was not studied for the function of isozyme and enzymatic properties at an early stage of germination during growth. So, this study was undertaken to examine the changes of peroxidase and properties of two isozymes isolated during germination.

Materials and Methods

Plant materials

Seeds of mung bean(*Phaseolus aureus Roxb.*) were germinated for a different period in a growth chamber in the dark at 27°C with the application of tap-water 4 times a day after imbibition for 12 hours. Depending upon germination time, mung bean sprout was divided into 5 sections as classified in Table 1.

Table 1. The mung bean parts used for enzyme source during germination

Germination days	1	2	3	4, 5, 6, 7
Mung bean part*	C,R	C,H,R	L,C,H,R	L,E,C,H,R

* L : leaf, E : epicotyl, C : cotyledon, H : hypocotyl and R : root

Enzyme extraction and assay

Each enzyme source was ground in a mortar with 2.5 volumes (w/v) of phosphate buffer (pH5.5) and then was centrifuged at 10,000×g for 10 minutes. The supernatant was collected and referred to crude homogenate. Peroxidase was assayed by the procedure described in the Worthington enzyme manual[28].

The standard assay system contained 0.492 mM 0-dianisidine, 0.192mM H_2O_2, 0.1ml enzyme solution, and 46.8mM phosphate buffer (pH5.5) with final volume of 3.2ml. Shimadzu-UV 200 spectrophotometer was used to measure the rate of decomposing of peroxide at 436nm. One unit of the enzyme activity is that amount of enzyme decomposing 1 μmole of peroxide per minute at 30°C, and the specific activity was expressed as units of enzyme activity was expressed as units of enzyme activity per mg protein.

Isolation and purification of isozyme

The crude homogenate of root parts germinated for 3 days was purified partially by the modified procedure of Takeo et al.,[20] Kokkinakis et al.,[22] and Claiborn et al.[29]

The crude homogenate was subjected to fractional precipitation with saturation of ammonium sulfate from 30 to 80%. The precipitation was collected and dialyzed against 10mM Tris-HCl buffer (pH8.2), insoluble matter being spun off. The supernatant was passed through a Sephadex G-75 column (2.0×43.0cm) and was eluted with 10mM Tris-HCl buffer (pH 8.2). The flow rate was 36ml per hour and the effluent volume was 3ml.

The fractions containing high enzyme activity were collected and applied to a column of DE-AE-cellulose. The concentrated solution was fractionated on a DEAE-cellulose column(1.5×23.0cm) which was preequilibrated with Tris-HCl buffer(pH8.2). Then the column was eluted by a linear gradient with NaCl (from 0 to 1.0 M) in the same buffer. The flow rate was 45 ml per hour and the effluent volume was 3ml. Eluates were monitored for protein at 280nm. All the procedure involved in column chromatography were carried out at 4°C.

Protein determination

Protein concentration was measured by means

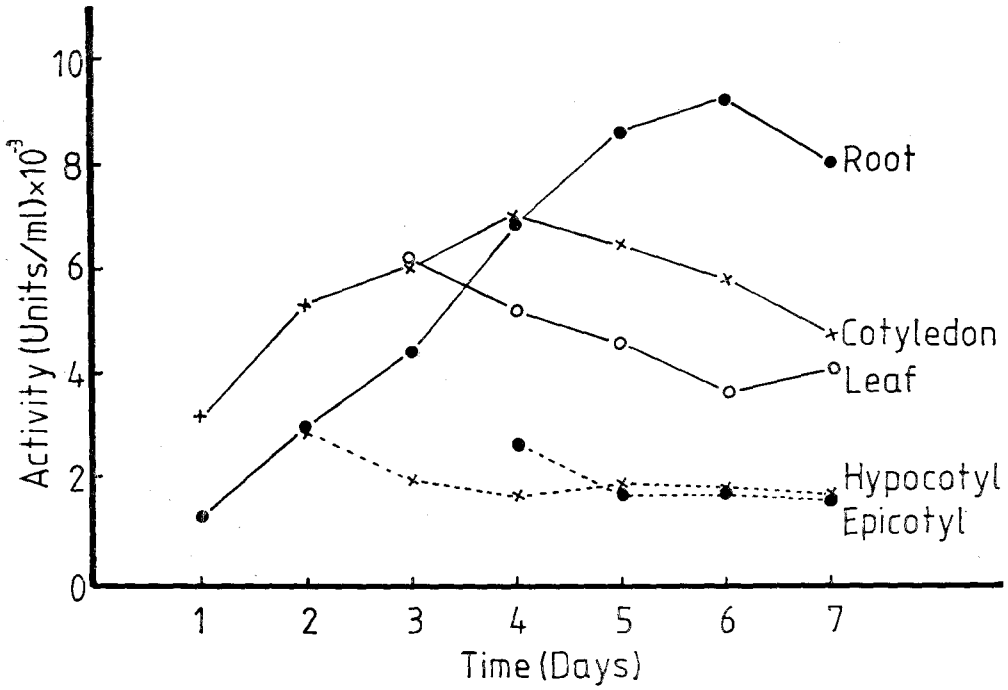

Fig. 1. Time course of peroxidase activity during germination of mung bean.

of Lowry et al.[30] using crystalline bovine serum albumin as a standard.

Polyacrylamide disc gel electrophoresis

Disc electrophoresis in polyacrylamide gel was performed as described by Davis[31] using 6% polyacrylamidegel. Electrophoresis was carried out at a constant current(3mA/tube) with bro mophenol blue as a migration marker. The localization of peroxidase activity band appeared when the electrophorized gels were soaked in 1.0M acetate buffer (pH5.0) at 25°C for 30 minutes and were incubated with the incubation medium (Table 2) at 30°C for 1 hour as described by Tao and Khan.[32]

Table 2. Incubation medium for staining of peroxidase activity band

Reagents	
Potassium phosphate buffer(pH 5.5)	46.8mM
Hydrogen peroxide	0.192mM
0-Dianisidine	0.492mM

Results and Discussion

Changes in peroxidase activity

As shown in Fig. 1, the enzyme activities in cotyledon and root showed a tendency to increase at an early stage of germination and were found to be maximal on the 4th day and 6th day, respectively. As mung bean developed, the crude homogenate of hypocotyl and epicotyl showed the continuous decline in the enzyme activity. In particular, enzyme activity in root was higher than those in any other parts at 4th day of germination. But the protein contents of crude homogenates declined continuously during germination (Table 3).

These results suggest that at an early stage of germination the peroxidase decomposes the abscisic acid and ethylene[32,33] which inhibit the growth of root which undergoes vigorous biochemical changes.

The distribution of peroxidase isozymes was determined by polyacrylamide disc gel electrop-

Table 3. Changes in protein content (mg/ml) during germination of mung bean

Time(days) / Parts	Protein content(mg/ml)						
	1	2	3	4	5	6	7
Root	2.71	2.10	1.65	1.42	1.34	1.11	0.93
Hypocotyl		1.96	1.87	1.34	1.27	0.88	0.84
Cotyledon	11.72	8.97	8.81	7.90	4.74	2.77	2.33
Epicotyl				3.74	2.16	1.83	1.41
Leaf			13.31	12.75	11.07	10.62	8.81

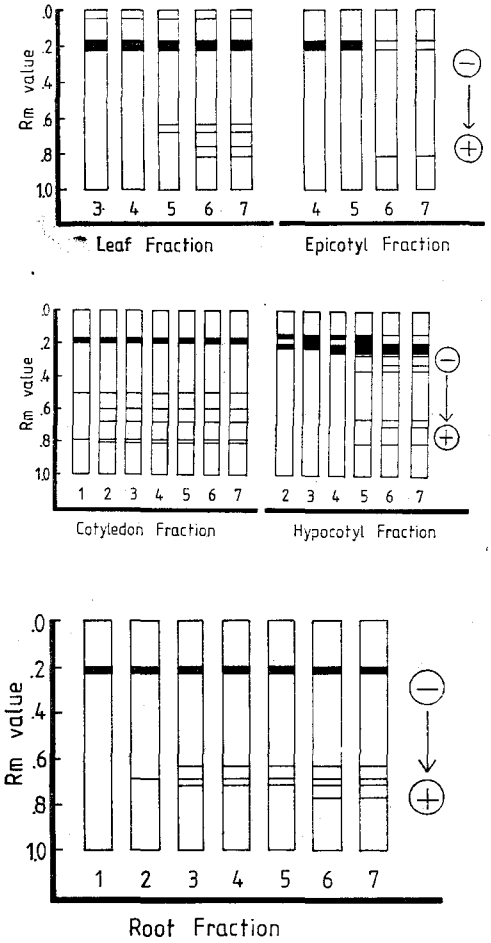

Fig. 2. Changes in peroxidase activity band from mung bean seedling. The 1, 2, 3, 4, 5, 6, 7 indicate germination times (Days).

horesis. Its profile was shown in Fig. 2. The number of isozymes at an early stage of germination was shown to be a few and gradually increased afterwards as germination went on. The number of isozymes increased until the 6th day after germination in leaf, epicotyl, hypocotyl and root, and did not change thereafter. This results suggested that the isozyme is necessary and its role is unique to growth and differentiation of plant. And also, regardless of the increase and decrease in enzyme activity, the number of isozyme increased as germination proceed, being the most at the 6th day of germination. The major band occurred at Rm value of 0.22 in all parts of mung bean. As compared with reports of Jun et al.[34] using guaiacol as hydrogen donor, the enzyme activity was relatively high. Some difference was observed in the isozyme pattern, but the Rm value of the main band was similar to that of Jun et al. The enzyme was also more sensitive to 0-dianisidine than to guaiacol as hydrogen donor. It is suggested that these results are due to the substrate specificity.

In electrophoretic study, we have observed that root have had higher enzyme activity and larger isozyme number than other parts of mung bean. So we tried to isolate of two enzyme activity bands(isozyme) among five bands at 3th day of germination in root. The Rm values of two bands have 0.22 and 0.62∼0.70, respectively.

Enzyme purification

Mung bean roots germinated for 3 days were subjected to isozyme purification in order to establish the purification procedure for pero-

Table 4. Summary of purification of peroxidase from roots of mung bean seedling grown for 3 days

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	124.64	455310	3742	100	1
30~80% $(NH_4)_2SO_4$	35.52	333521	9389	73	3
Sephadex G-75	25.79	328114	12717	72	4
DEAE-cellulose					
fraction I (No 11~13)	0.61	36058	58919	8	16
fraction II (No 19~21)	1.29	4185	3245	1	1

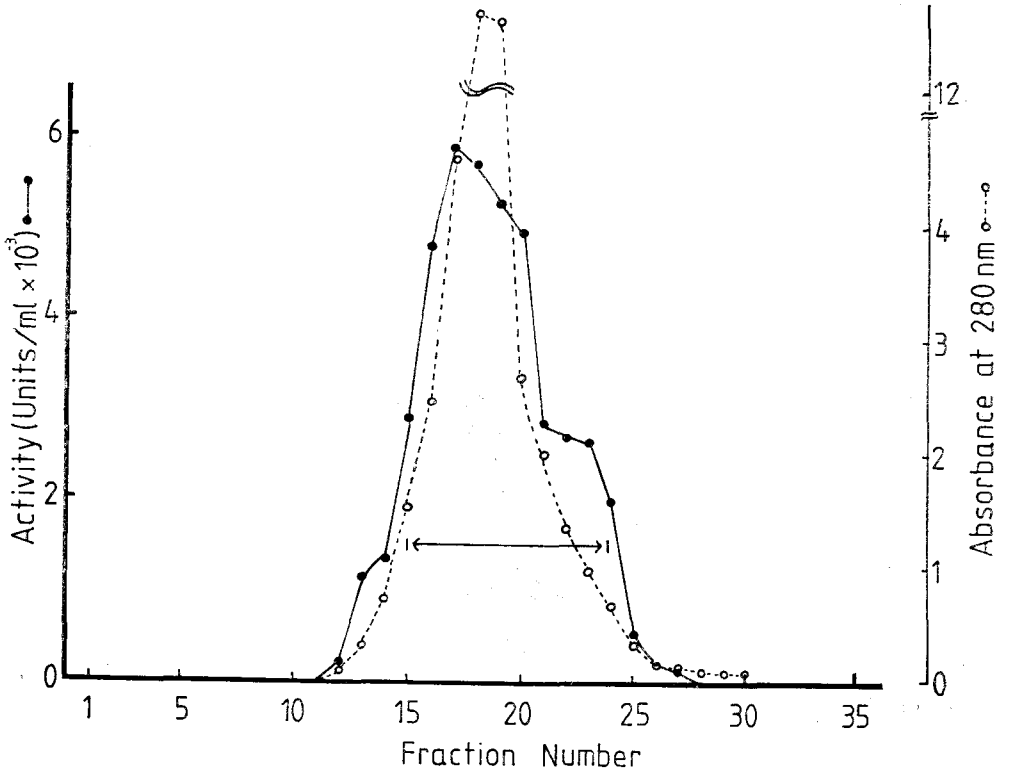

Fig. 3. Sephadex G-75 gel filtration of the enzyme fraction obtained from 30~80% saturated $(NH_4)_2SO_4$ fractionation. The column size was 2.0×43.0cm. Fraction volume collected was 3ml as a flow rate of 36ml per hour.

xidase and to characterize the enzyme in terms of kinetic behavior, metal ion specificity and heat stability, etc.

The enzyme purification procedure included ammonium sulfate fractionation, gel filteration on Sephadex G-75, and ionxchange on DEAE -cellulose.

Table 4 summarizes the results of purification procedures for mung bean peroxidase. The final preparation of the isozyme A was purified about 16 fold with 8% recovery, but the purity of the isozyme B was not increased and its yield was low. Because the isozyme B was originally present in small amount in the crude extract, a low yield would be expected.

The bulk of peroxidase (30ml), which was

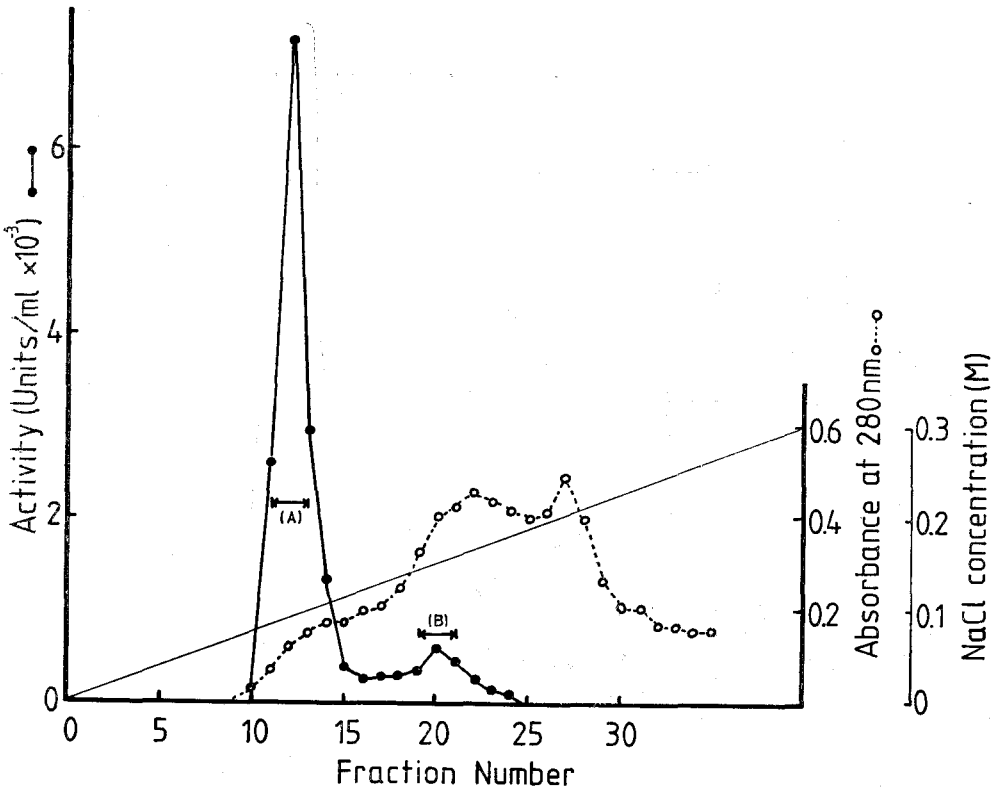

Fig. 4. Column chromatogram of the active fraction from Sephadex G-75 gel filtration on DEAE-cellulose. The column size was 1.5×23.0cm. Fraction volume collected was 3ml as a flow rate of 45ml per hour.

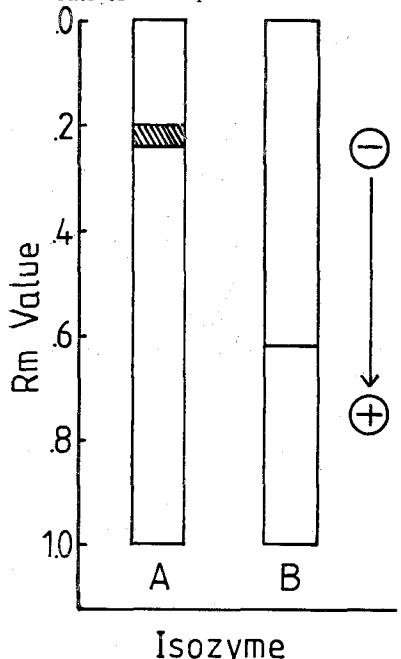

Fig. 5. Disc gel electrophoresis of the partially purified two isozymes, fraction I(A) and fraction II (B).

eluted from the Sephadex G-75 column (Fig. 3), was applied on DEAE-cellulose column. On DEAE-cellulose column chromatography, the activity was observed in two peaks (the first peak, from 33ml to 42ml and the second peak, from 57ml to 63ml) (Fig. 4). In our experiment, the first peak was named fraction I or isozyme A and the second peak fraction II or isozyme B. When the two fraction were electrophorized, each of them had only single activity band, its Rm value being 0.22 and 0.62, respectively, being same to those of crude homogenate electrophorized (Fig. 5).

Enzyme properties

Table 5 shows the effect of divalent cations on peroxidases. In 1 mM concentration, all the divalent cations except Hg^{++} employed were found to have remarkable activation effects on

Table 5. Effects of divalent cations on peroxidase activities.

Enzyme	Cation conc.	Cation							
		$CaCl_2$	$MnCl_2$	$CoCl_2$	$MgCl_2$	$CuCl_2$	$FeCl_2$	$HgCl_2$	None
Isozyme A	1mM	143	110	125	131	129	105	41	100
	10mM	94	92	105	108	72	96	72	100
Isozyme B	1mM	119	105	119	131	138	113	35	100
	10mM	94	75	107	107	82	95	82	100

* Enzyme activity of the control was taken as 100.

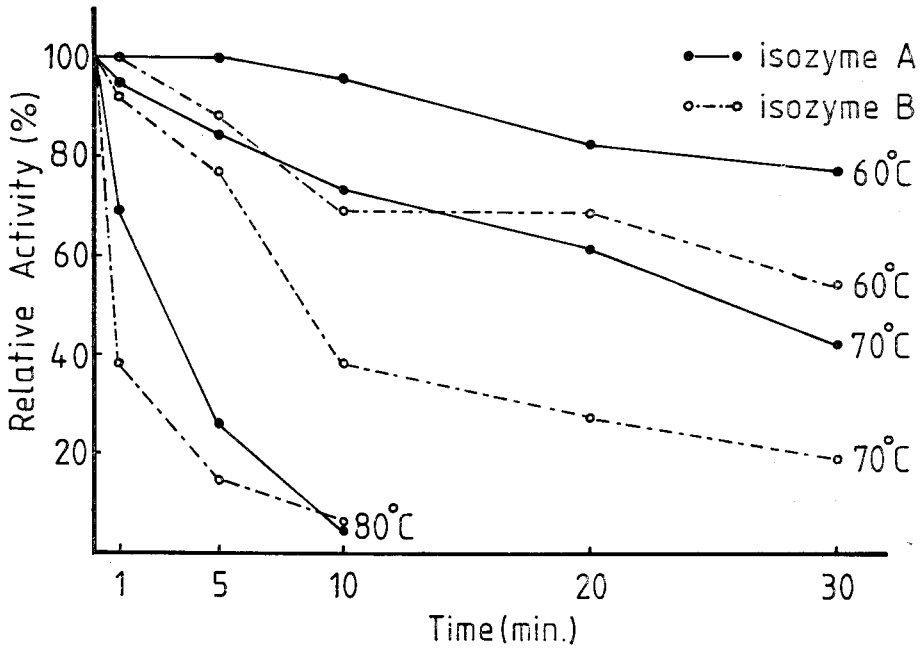

Fig. 6. Effects of temperature on the stabilities of peroxidase isozymes A and B.

both isozymes. But almost all of the cations except Co^{++} and Mg^{++} inhibited the enzyme activity at 10mM concentration.

The effect of heat treatment on peroxidase was investigated at pH 5.5 under various temperatures (Fig. 6). Inactivation of peroxidase occurred upon exposure to temperature higher than 60°C, as shown in the case of peroxidase isolated from Japanese radish.[35] When incubated for 10 minutes at 70°C, the remaining activity of isozyme A dropped nearly to 80% and isozyme B to 40%. The two isozymes (A, B) were completely inactivated at 80°C for 10 minutes. These results show that two isozymes are considerably stable on heat.

Table 6. Summary of optimal, pH, temperature and Km value for isozymes A and B.

	Isozyme A	Isozyme B
Optimal pH	5.6	5.6
Optimal temperature(°C)	65	70
Km for H_2O_2(mM)	0.28	0.23
Km for 0-dianisidine (mM)	0.071	0.052

Table 6 summarizes the results of optimal temperatures, pHs and Km values for peroxidases. Each isozyme showed maximal activity over

a broad pH range using McIlvaine buffer (citrate phosphate buffer).

The optimal pHs of peroxidase isozyme A and B were found to be 5.6, with less than 80 % of the maximal activity exhibited at pH 4.6 and 6.2 for isozyme A and at pH 5.0 and 5.8 for isozyme B. With 0-dianisidine as substrate, Balasimba et al.,[24] Shannon et al.[21] reported a similar pH optimum for fenugreek, horseradish peroxidase. The optimal temperatures of isozymes tested within the temperature range between 30°C and 80°C were 65°C for isozyme A and 70°C for isozyme B.

Classical kinetic analysis was performed on our isolated mung bean peroxidase fraction that contained two isozymes. The Km value was estimated by a double reciprocal plot suggested by Lineweaver-Burk.[36] The Km value of isozyme A for both H_2O_2 and 0-dianisidine was larger than that of isozyme B. The apparent Km values of isozyme A and B were 0.28, 0.23mM for H_2O_2, and 0.071, 0.052 mM for 0-dianisidine.

On the basis of our study for enzymatic properties, optimal pH, temperature, heat stability and Km value etc., the isozyme A probably corresponds to isozyme B of Balasimba et al.,[24] and isozyme B to isozyme D.

Abstract

The changes in peroxidase activity and its isozyme pattern in the different parts of mung bean sprout were investigated; The enzyme activity in cotyledon and root showed a tendency to increase at an early stage and then decreased gradually as germination continued. However, the crude homogenate of epicotyl and hypocotyl showed a continuous decline in the enzyme activity. In particular, the enzyme activity of the root was 1.5~3.5 times higher than that of other parts.

Gel electrophoresis of the crude homogenate revealed that the number of isozyme in every part of the mung bean sprout increase during germination up to 6th days.

Two isozymes from root were partially purified by ammonium sulfate fractionation, gel filtration by Sephadex G-75 and DEAE-cellulose column chromatography. One of the isozymes (A) was purified 16-fold by the present procedure, but the purity of the other isozyme (B) was not increased, significantly.

Isozyme A was the most active at 65°C and isozyme B at 70°C, while both isozyme (A, B) have a optimal pH of 5.6. The Km values of isozyme A and B for 0-dianisidine as a hydrogen donor determined to be 0.071 mM and 0.052mM, respectively, and those for isozyme A and B using H_2O_2 as a hydrogen acceptor were 0.28mM and 0.23mM, respectively.

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