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Purification and Characterization of Polyphenol Oxidase in Sweet Potato (Ipomoea batatas)

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고구마 Polyphenol Oxidase의 정제 및 특성

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Abstract

The present work was undertaken to investigate the purification and characterization of polyphenol oxidase (PPO ; EC 1.10.3.1) in sweet potato, particularly the number of PPO isozymes, and PPO properties such as pH optimum, heat stability, substrate specificity, kinetics, and inhibitor studies. The purification achieved was 23.1 fold from crude extract with a yield of 41.5%. Eight PPO isozymes and twelve PPO isozymes were detected by disc polyacrylamide gel electrophoresis and isoelectric focusing, respectively.

The specific activity of each isozyme separated by isoelectric focusing was in the range of 6,000~46,700 U/mg protein. This enzyme was stable below 65℃ and the pH optimum of PPO occurred at 6.0-6.5. The substrate specificity of sweet potato PPO showed the high affinity toward the odiphenolic compounds. Km and Vmax for catechol were found to be 6.7 mM and 20△A/min, mg protein, respectively. Inhibitor studies indicated that dithiothreitol was the most potent among the inhibitors used in the present work.

Introduction

The oxidative reaction associated with the undesirable darkening of damaged tissue in fresh fruits and vegetables is catalyzed by the enzyme, polyphenol oxidase(PPO ; O-diphenol : O_2 oxidoreductase, EC, 1.10.3.1. ; also known as phenolase, phenol oxidase, catechol oxidase, tyrosinase, chlorogenic acid oxidase, and O-diphenol oxidse).

Polyphenol oxidase is a copper containing enzyme which catalyzes either one or two reactions involving molecular oxygen. The first type of reaction is ortho-hydroxylation of monophenols leading to formation of O-dihydroxy compound.

The second type of reaction is oxidation of O-dihydroxy compund to quinones, which either react with themselves or with other phenolics to form the brown pigment called melanin. The degree of some varieties was correlated with the tannin content of the ripe fruit and, in some cases, with the total PPO activity. PPO has also been implicated in the resistance of plants to various microorganisms. Szent-Gyorgi and Vietorsz[1] have concluded that quinones produced by the intensive oxidative operation of phenolase in injured tissue are toxic to invading pathogens. Upon infection, the activity and amount of phenolase in plant generally increases.

This enzyme is widely distributed throughout the plant kingdom, but its activity is particularly

high in fruits and vegetables. PPO has been purified and studied from peaches, cranberries, banana, pears, avocadoes and apples.[2~7]

Recent investigators[8~10] have found that instead of there being only a single phenol oxidizing enzyme per species, multiple forms could be separated on the basis of molecular weight or electrophoretic mobility, or both. Robb et al.[8] separated multiple forms of broad bean PPO, having similar molecular weight and specificities, by starch gel electrophoresis. Constantinides et al.[9] used polyacrylamide gel electrophoresis to separate multiple forms of PPO from mushroom, apples and potatoes,and the reported number of multiple forms of PPO ranges from 2 in melanoma to as many as 11 in potato.[9]

The PPO of sweet potato has been studied by several groups. Arthur, Jr. and Mclemore[11] reported the activation of PPO and discoloration of sweet potato during processing. They also studied the interaction between copper ions and PPO oxidized substrates.[12] Hyodo and Uritani[13] isolated three PPO which had the different mobilities on the gel electrophoresis and they als oreported some properties of PPO in healthy and infected sweet potato tissue.[14~15] However, there are few studies on the further purification of sweet potato PPO, the number of its isozymes.

The present study was undertaken to investigate the partial purification of PPO in sweet potato, the number of its isozymes. In addition, some characterizations such as the thermal inactivation, pH optima, substrate specificity, kinetics and inhibitor studies of PPO in sweet potato were also studied.

Materials and Methods

Materials

Sweet potatoes(Ipomoea batatas, variety, Hong-mi) were obtained at rocal market in Pusan and the most fresh ones were used as samples. Pharmalyte(pH range 3−10) was purchased from Pharmacia Fine Chemicals Inc. DEAE−cellulose (medium mesh and ion−exchange capacity of 9.2meq / gm), Acrylamide, N,N'-methylene-bis-acrymide and pI calibration kit [amyloglucosidase (Aspergillus oryzae, pI 3.55), trypsin inhibitor(-soybean, pI 4.55), β-lactoglobulin A(milk, pI 5.13), carbonic anhydrase B(bovine erythrocyte, pI 5.65) carbonic anhydrase B(human erythrocyte pI 6.57), myoglobin(horse heart, pI 6.76, 7.16), L-lactic dehydrogenase(rabbit muscle, pI 8.30, 8.40 and 8.55) and trypsinogen(bovine pancrease, pI 9.30] were obtained from Sigma Chemical Co. All other chemicals were reagent grade.

Purification procedure

All steps for the purification of PPO from sweet potato were carried out as following procedures at about 4℃ unless otherwise mentioned.

Extraction of the active enzyme : Fresh sweet potatoes were washed with deionized water and peeled immediately. About 150gm of sweet potato peels were homogenized at full speed for 2.5min in Omni-mixer(Sorvall, Model 17105) with 300ml of ice-cold 0.1M sodium phosphate buffer(pH6.2) containing 0.03M ascorbic acid and 0.5% polyethylene glycol(M.W. 6,000−7,000) to prevent browning. The homogenate was filtered through a double layer of cheesecloth with a glass wool plug in the funnel and filterate was centrifuged at 11,000 g for 40min in refrigerated centrifuge (Sorvall, Model RC−5B) and the collected supernatant fraction was used as crude enzyme extract for PPO purification.

Fractionation by ammonium sulfate : To crude enzyme extract, 62.7gm solid 62.7gm solid ammonium sulfate was added with gentle stirring over a period of 30 min to 35% saturation. After 2 hours, the precipitate was removed by

centrifugation at 11,000 g for 30min. Another(157. 8gm) solid ammonium sulfate was added to final 70% saturation and stood overnight. The precipitate collected by centrifugation was suspended in 35ml of 1mM sodium phosphate buffer(pH 6.2) and dialyzed against the same buffer for 48 hours. The dialysate was recentrifuged at 20,000 g for 40min.

3. DEAE-cellulose chromatography : The pre vious supernatant (50ml) was absorbed to DE AE -cellulose column (2.5×26cm) previously eq uilibrated with 1mM sodium phosphate buffer (pH 6.2) and washed with two bed volumes of the same buffer to remove the bulk of inactive protein. The column was eluted with a 500ml linear gradient from 1 to 250mM sodium phosphate buffer (pH 6.12) at flow rate of 37ml / hr. Fractions of 4.8ml were collected with fraction collector(Instrumentation Specialties Company, Model 328) with a UV monitor system, and each fraction was assayed for its enzyme activity. The enzyme was eluted as two major peaks, from 90 to 110mM sodium phosphate(peak A) and from 110 to 150mM sodium phosphate(peak B). Each peak was pooled and dialyzed against 1mM sodium phosphate buffer (ph 6.2) and used for all further studies

Measurement of enzyme activity

The enzyme activity was determined by measuring inital rates of the increase in absorbance at 420 nm with spectrophotometer(Gilford, Model 2600) at 30℃ by the methods of Galeazzi et al.[16] and Park et al.[17] with modification. The sample cuvette contained 2.9ml 10mM catechol in 10mM sodium phosphate buffer(pH 6.2) and 0.1ml enzyme soltion. The reference cuvette contained 2.9ml 10mM catechol in 10mM sodium phosphate buffer (pH 6.2) and 0.1ml deionized water under these condition. One unit of PPO enzyme was defined as the amount of potency enzyme causing a change in absorbance by 0.001 / min. The straight line section of the activity curve was used to express the enzyme activity.

Protein concentration was determined by the method of Lowry et al[18] using bovine serum alaumin as a standrd

Disc polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed by the method of Davis[19] using the tube(5mm i. d.) filled with 6.5cm separating gel(7%) and 1.5 cm stacking gel(1.5%). The enzyme solution was-mixed with 40% sucrose and layered on top of stacking gel(1.5%). The starting pH was 8.3 and the running pH was 9.5. Electrophoresis was carried out at 4℃ at a constant current of 2mA / tube with the anode at the bottom. Electrophoresis was stopped when bromophenol blue(BPB), tracking dye, reached to 1cm from the end of the tube. Active band was detected by a modification of Van Loon.[20] The gels were incubated in 10mM sodium phosphate buffer(pH 6.2) containing 10mM catechol and 0.05% p-phenylenediamine as coupling agent for 10-30 min at 30℃. For stabilizing the color of the stained band and decreasing the no nspecific background color,the gels were stored in 30% ethanol solution. A part of the gels were stained for protein with 0.1% Coomassie Blue R -250 in 7% acetic acid, 25% ethanol, and 68% water.

Isoelectric focusing(IEF)

IEF in polyacrylamide gel rods was carried out as described in the laboratory techniques of Pharmacia Fine Chemicals.[21] The tubes(5mm i.d.) was filled to 8cm with polyacrylmide(T 5%, C3%), containing 6.25% pharmalyte(pH range 3-10). The gel was polymerized by incubating at room temperature for 16 hours. After gel polymerization, the gel tubes were filled to the top with a solution consisting of 6.25% pharmalyte and 15% sucrose, and prefocused to remove the contaminants and generate the pH gradient for 20-30 min at 1mA /

tube. The anode contained 0.4% H_2SO_4, and the cathode 0.4% ethanolamine. The sample containing 15% sucrose, 6.25% pharmalyte and methyl red was applied. IEF was conducted at a constant current of 1mA / tube, setting a maximum voltage of 500 volts(for 3−3. 5 hours). Following electrofocusing, one gel was stained for PPO activity and another gel for protein staining was fixed in fixing solution(30% methanol, 3.4% sulphosalicylic acid and 11.5% trichloroacetic acid; V / W / W / V) for 1 hour and stained with Coomassie Blue R−250 by the method as described under gel electrophoresis. pI calibration kit used was amyloglucosidase (Aspergillus oryzae, pI 3.55), trypsin inhibitor(soybean, pI 4.55), β−lactoglobulin A(milk pI 5.13), carbonic anhydrase B(bovine erythocyte, pI 5.65), carbonic anhydrase B(human erythrocyte, pI 6.57), myoglobin(horse heart, pI 6.67, 7.16), L−lactic dehydrogenase(rabbit muscle, pI 8.30, 8.40 and 8.55) and trypsinogen(bovine pancrease, pI 9,30).

Thermal inactivation of enzyme

Thermal inactivation studies were done 65, 70, 80 and 85℃. 5ml portion of PPO solution was incubated at each of the above temperature and the samples were removed at various time intervals and immediately cooled by immersion in a prechilled test tube on ice. The initial rate of PPO for the samples was measured as described in the enzyme assay.

pH optimum

pH optimum of PPO was examined by measuring initial rate of the enzyme activity as described in the enzyme assay using 10mM sodium acetate buffer(pH 4.0−5.5), 10mM sodium phosphate buffer (pH 5.5−7.5) and 10mM Tris−HCI buffer(pH 7.5−9.0)

Substrate specificity.

The PPO activity as a function of substratewas determined with eleven different substrate, consisting of monophenols,ortho and meta diphenols, and trihydroxy phenolic compounds. The substrate concentration was 10mM for all except tyrosine which was 2.5mM due to limited solubility and the substrate specificity was expressed as percentage activities compared to the activity with catechol.

Inhibitor studies

The effects of various levels of nine different inhibitors on the activity of PPO were determined in 10mM sodium phosphate buffer (pH 6.2) with 10mM catechol as substrate. Inhibitory effects were expressed as relative percentage of inhibition compared to the activity of the enzyme without inhibitors.

Results and Discussion

The results of a representative purification experiment of the sweet potato PPO in each procedure were summarized in Table 1. There was a slight increase in the specific activity when homogenate

Table 1. Purification of PPO in sweet potato

Step of purification	Total volme (ml)	U / ml / min ($\times 10^3$)	Activity Total U ($\times 10^4$)	Protein (mg / ml)	Specific activity (U / mg protein) $\times 10^3$	Yield (%)	Purification fold
Buffer extract	300	4.4	132	5.88	0.748	100	1
35-70%$(NH_4)_2SO_4$ fractionation	50	12	60	8.32	1.442	45	1.93
DEAE−cellulose	82	6.7	54.9	0.387	17.312	41.5	23.14

One unit=△A of 0.001 at 420nm per min.

was fractionated with 70% ammonium sulfate.

The DEAE−cellulose column chromatography resulted in 23.1 fold increase in the specific activity of the enzyme with recovery of the enzyme activity of 41.5%.

A typical pattern of the DEAE−cellulose column chromatography is shown in Fig. 1. Two major peaks of activity appeared with more activity in peak B than A. PeakA was eluted from 90 to 110mM sodium phosphate and peak B from 110 to 150mM sodium phosphate. Fig. 2 represents the gel electrophoretic analysis of the PPO eluted in the fraction tubes of peaks A and B as shown in the elution profile of Fig. 1 and a similar electrophoresis pattern was detected in either peak after incubation with catechol substrate. Eight active bands were revealed and the intensity of the band was f,g>d,e,h>a,b,c. The elution profile of this enzyme on DEAE−cellulose column and the results by means of subsquent gel electrophoretic analysis were different from those of PPO from sweet potato of different variety. Hyodo and Uritani[13] reported that the PPO from sweet potato (variety, Norin 1) was resolved into three peaks on DEAE−cellulose chromatography and that each peak showed up as a single active band with different mobility for the bands of each peak by electrophoresis. But in this study, the enzyme was resolved into closely associated peaks (Fig. 1) and each peak showed eight active bands in gel electrophoretic analysis (Fig. 2).

Isoelectric focusing was performed to resolve the PPO isozymes in sweet potato according to pI. Fig. 3 demonstrated the isozyme pattern of sweet potato PPO when equal amount of enzyme solution from peaks A and B (Fig. 1) were applied separately to isoelectric focusing gel. Twelve PPO isozymes in the range of pI 3.2−9.6 were detected from the sweet potato (Fig. 3). Isoelectric focusing results of peaks A and B showed the similar isozyme pattern in either peak, but slight differed in isozyme activity.Isozymes with higher pI than neutral pH were predominant in peak A but ones with lower pI than neutral pH in peak B. Lane 3 in Fig. 3 represents the isozyme pattern of the combined fraction of the peaks A and B,and twelve active bands were also detected. Since the isozyme pattern of combined fraction was simlar to that of respective peaks A and B, the combined fraction was used in the present work. By comparison with the pI of marker protein, the pI of isozymes was evaluated

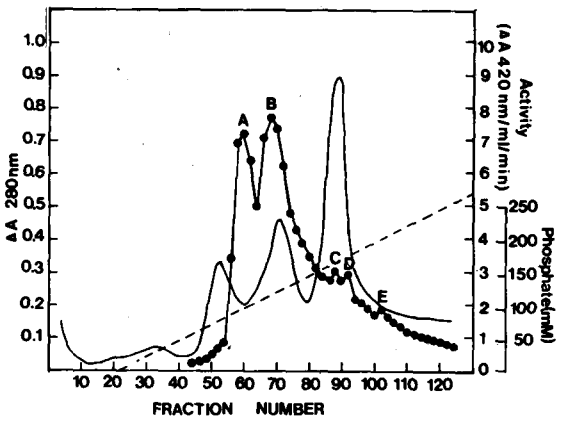

Fig. 1. Elution profile of the sweet potato PPO through a DEAE−cellulose column.
Sodium phosphate ;------ Protein concentration; A 280 ———, Enzyme activity; A 420 •———•

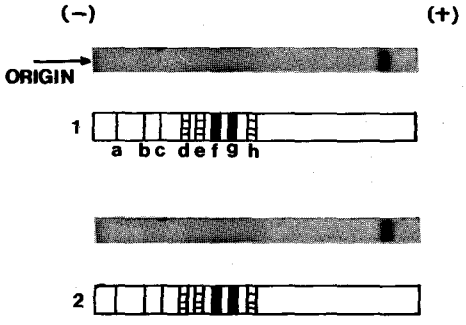

Fig. 2. Polyacrylmide gel electrophoresis of sweet potato PPO. The PPO isozymes with catechol activity are designated as "a" through "h".
Lane 1 ; electrophoretic analysis of peak A(see Fig. 1)
Lane 2 ; electrophoretic analysis of peak B
Intensity of the bands : high ■■■ ; medium □□□ ; low ———

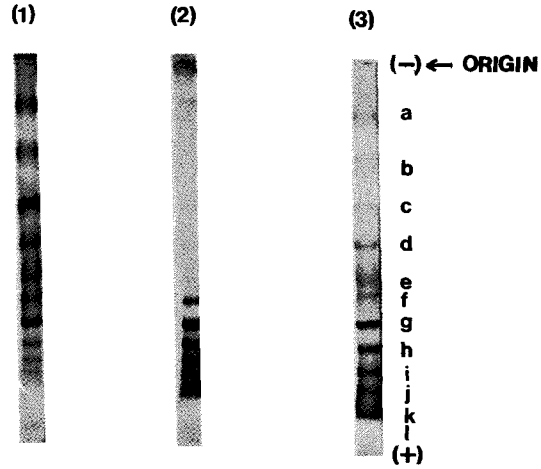

Fig. 3. Resolution of the sweet potato PPO isozymes designated as "a" through "I" by isoelectric focusing ; using pharmalyte (pH range 3−10).
Lane 1 ; Isozymes pattern of peak A (see Fig. 1)
Lane 2 ; isozymes pattern of peak B
Lane 3 ; isozymes pattern of the combined fraction of peak A and B

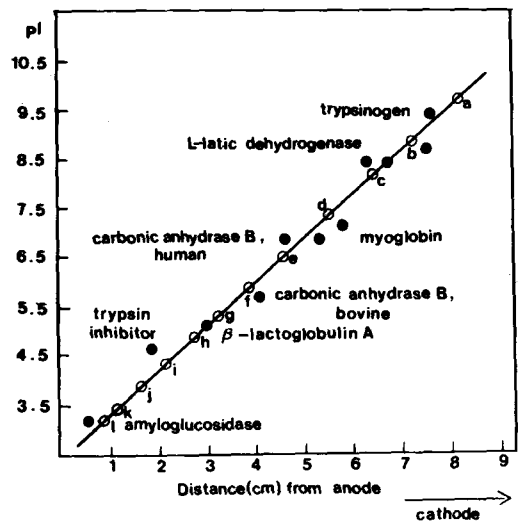

Fig. 4. pI determination of the sweet potato PPO isozymes designated as "a" through "I". the pI of these were evaluated to be 9.6, 8.7, 8.0, 7.2, 6.4, 5.8, 5.3, 4.7, 4.3, 3.8, 3.4 and 3.2, respectively. pI calibration kit (black circles) described in method was used for the pI determination.

for "a"through "1" to be 9.6, 8.7, 8.0, 7.2, 6.4, 5.8, 5.3, 4.7, 4.3, 3.8, 3.4 and 3.2, respectively (Fig. 4). Kahn[22] reported that seven PPO isozymes were detected from avocado by either gel electrofocusing or disc polyacrylamide gel·electrophoresis. In this experiment, eight PPO isozymes were detected from sweet potato by disc polyacrylamide gel electrophoresis (Fig. 2) and twelve PPO isozymes by gel electrofocusing(Fig. 3). These results suggest that PPO from sweet potato results in more isozymes by gel electrofocusing than gel electrophoresis and it is also found that there are isozymes in PPO from sweet potato with the same charge density but different pI.

On the basis of scanning densitometry of the stained gel, the determinations of relative enzyme activity and protein of each isozyme were carried out after isoelectric focusing. The protein concentration and activity of enzyme sloution applied to gel were accurately determined. Fig5 shows a densitometric tracing of the gel. Rotation of the gels about their long axes made no changes in the densitometric tracings. As shown in Table, 2, the specific activity of the each isozyme was determined for "a" through "I" bands to be 15, 800, 6,000, 13,400, 15,500, 9,200, 22,300, 46,700, 25, 900, 37,700, 32,900, 17,000, 27,700 units / mg protein, respectively. f,g,h,i,j and I bands of isozymes showed relative higher specific activity than that of eluate from DEAE−cellulose chromatography. The "g" band compared to others represented the hightest specific activity.

The results of thermal inactivation at 65℃, 70℃, 80℃ and 85℃ are shown in Fig. 6. As expected, the rate of thermal inactivation was greater with increasing temperature and followed first order reaction. This enzyme was stable below 65℃, and approximately 50% of the enzyme activity was lost after heating for 15, 5 and 1 min at temperature of 70℃, 80℃, 85℃, respectively and the enzyme activity was lost completely after heating for 5. 5 min at 85℃. While the PPO from pears was stable below 60℃ and lost a half of the original activity at 80℃ in 2 min. The PPO from sweet potato showed some higher heat stability than

Table 2.

The results of densitometric tracing of the stained gel and the specific activity of the each isozyme. Amount ofenzyme solution applied to isoelectric focusing gel was 38.7 μg protein (corresponding to 670 activity units).

Isozymes		a	b	c	d	e	f	g	h	i	j	k	l
Gel stained with substrate	Ratio of total area (%)	4.0	3.3	3	5.1	4.8	7.0	16.7	15.1	13.5	10.8	5.1	12
	Relative amount of activity (U)	26.8	22.1	20.1	34.2	32.2	46.9	112	101	90.4	72.3	33.5	80.4
Gel stained with Coomissie Blue R-250	Ratio of total area (%)	4.4	9.6	3.9	5.6	9.0	5.4	6.2	10.1	6.1	5.8	5.1	7.5
	Relative amount of protein (mg) $\times 10^3$	1.7	3.7	1.5	2.2	3.5	2.1	2.4	3.9	2.4	2.2	1.97	2.9
Specific activity (U/mg protein) $\times 10^3$		15.8	6.0	13.4	15.5	9.2	22.3	46.7	25.9	37.7	32.9	17.0	27.7

the PPO from pears and the difference in heat stabilities between these two enzyme might be due to the difference in their physico-chemical properties.

The pH optimum of this enzyme generally showed up in the range of pH 6.0-6.5 (Fig. 7), and was similar to that of PPO from sweet potato of different variety.

It was reported that PPO in fruits such as d' Anjou pears[5], apples[24] and clingstone peaches[2] was the most active at neutral range of pH. It is therefore clear that more activity of sweet potato PPO over other fruits is in more acidic range of pH.

In order to investigate the substrate specificity of this enzyme, various phenol compounds were used as substrates. The monophenols, ortho and m-phenols, and trihydroxy phenols selected as the substrates were as follows ; catechol, 4-met-

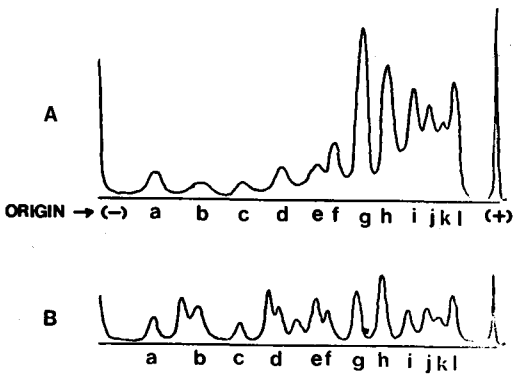

Fig. 5. Densitometric tracing of the sweet potato PPO isozymes which are designated as "a" through "I" separated by analytical isoelectric focusing.
A : densitometric tracing of the gel stained with catechol
B : densitometric tracing of the gel stained with Coomassie Blue R-250.

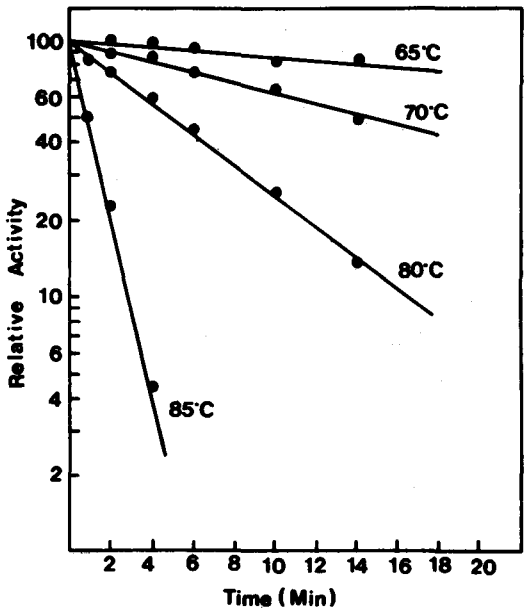

Fig. 6. Thermal inactivation of the sweet potato PPO

hylcatechol, DL−DOPA, dopamine, d−catechin, pyrogallol, gallic acid, L−tyrosine, p−cresol, phloroglucinol and resorcinol. Since catechol showed the highest activity for this enzyme, it was used as the standard for compairson. The relative activity for the various substrates was calculated compared to the activity with catechol. As shown in Table 3, sweet potato PPO was specific for o−diphenolic compounds, in particular for catechol, 4−methyl catechol, DL−DOPA and dopamine. This enzyme was not able to oxidize monophenols and m−phenols, but there was a trace of activity with trihydroxy phenols except gallic acid. These results indicate that sweet potato PPO is o−diphenol oxidase, and were in accordance with the result from pears PPO,[23] but differed from results of other researches.[25,26] They reported that some plant PPO, i. e mushroom[25] and potato[26] catalyzed both the hydroxylation of monophenols and the oxidation of o−diphenols.

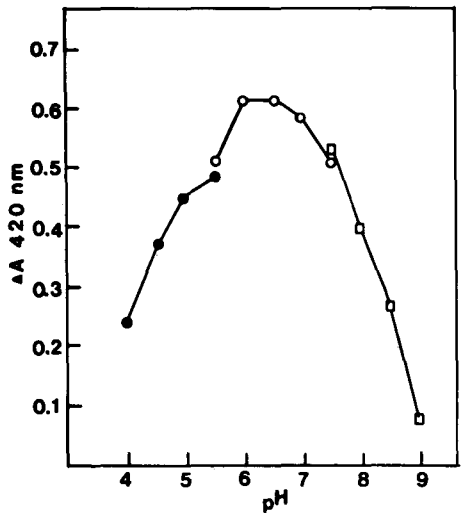

Fig. 7. pH optimum profile of the sweet potato PPO. 10 mM sodium acetate buffer (pH 4.0−5.5, •——•), 10mM sodium phosphate buffer (pH 5.5−7.5, ○——○), and 10mM Tris−Hcl buffer (pH 7.5−9.0, □——□ were used as substrate solution.

The kinetic parameter were determined at pH 6.2 at 30℃. Double reciprocal plot for catechol is shown in Fig.8, and Km and Vmax of the enzyme are 6.7mM and 20△A / min, mg protein, respectively. The km value of sweet potato PPO for the catechol was lower than that of Royal Ann

Table 3. Substrate specificity of sweet potato PPO on phenolic compounds.

Substrate	Concentration (mM)	Relative activity(%)
o−Diphenols		
catechol	10	100
4−methl catechol	10	54
DL−DOPA	10	30
dopamine	10	35
d−catechin	10	20
m−Diphenols		
phloroglucinol	10	0
resorcinol	10	0
Trihydroxyphenols		
pyrogallol	10	20
gallic acid	10	0
Monophenols		
L−tyrosine	2.5	0
p−cresol	10	0

Cherries PPO,[27] and this result indicated that the physical nature of sweet potato PPO is somewhat different from that of Royal Ann Cherries.

The effect of inhibitors at 0.5, 1.0 and 5.0mM concentration on sweet potato PPO was examined and the result is shown in Table 4. Among the compounds used in the inhibitor studies, EDTA was the least potent inhibitor. In contrast, dithiothreitol was the most potent inhibitor for sweet potato PPO, and this result was in accordance with Benjamin and Montgomery's work.[27]

It was reported that cysteine reduced the enzymatic browning in fruits by complexing with the formed quinone and by being a reducing agent.[28] In this study, cysteine was also found to be potent inhibitor for this enzyme.

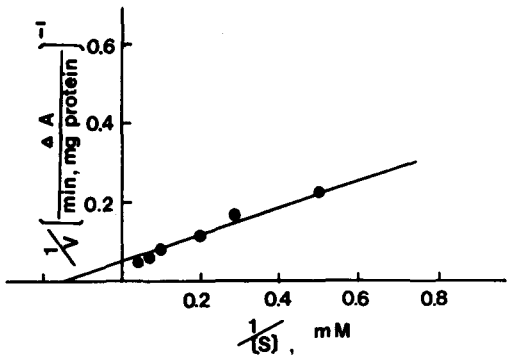

Fig. 8. Lineweaver-Burk plot of the sweet potato PPO activity. The reaction mixture contained 0.1ml enzyme solution, various concentration of catechol in 10mM sodium phosphate buffer (pH 6.2). Substrate concentrations ranged from 2 to 20 mM.

요 약

갈변반응에 관여하는 polyphenol oxidase(PPO : EC 1.10.3.1)를 한국산 고구마(Ipomoea batatas, var : Hong-mi)로부터 추출하여 ammoniun sulfate 분획 및 DEAE-cellulose column chromatography법에 의하여 정제한 결과, 효소활동도는 23.1배였으며 enzyme activity 수율은 41.5%이었다.

이 효소는 일반 전기 영동법에 의하여 8개의 isozymes 으로, 또한 isoelectric focusing에 의하여 pI가 각각 다른 12개의 isozymes으로 분리되었고 그 pI의 범위는 3.2-9.6이었으며, Isoelectric focusing에 의하여 분리된 각 isozyme의 specific activity는 6,000-46,700U / mg protein의 범위에 있었다.

고구마 중의 PPO는 65℃이하에서는 안정하였으며 85℃에서는 1분 가열에 의하여 약 50%의 효소활성이 상실되었고, pH optimum은 6.0-6.5이었다. o-diphenol이 이 효소의 가장 좋은 기질로서, 이 효소는 o-diphenolase임이 확인되었고, catechol에 대한 Km치는 6.7mM로 나타났다. 또한 이 효소에 대한 저해작용은 dithiothreitol, cysteine 및 ascorbic acid 순으로 크게 나타났다.

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