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# Purification and some properties of polyphenol oxidase from *Spuriopimpinella bracycarpa*

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**Abstract** : Three polyphenol oxidase(polyphenol oxidase Ⅰ, Ⅱ and Ⅲ) were isolated from the crude extract of a *Spuriopimpinella bracycarpa* by $(NH_4)_2SO_4$ precipitation and subsequent Sephadex G-150 chromatography. The final preparation thus obtained showed three peaks of enzyme activity. Optimum pH and temperature for the activity of polyphenol oxidase were 7.5 and 30℃, respectively. The enzyme was completely inactivated when it was treated at 70℃ for 30min and at 80℃ for 5min at pH 6.5. The enzyme was partially inactivated by ascorbic acid, glutathione and potassium cyanide (0.1mM), and was completely inhibited by L-cysteine, ascorbic acid, glutathione and potassium cyanide(0.5 and 1.0mM). The enzyme has good activity on catechol and 3,4-dihydroxytoluene but was strongly inactivated on pyrogallol, dopamine and DL-dopa. The Michaelis constant of the enzyme was 86.5mM with catechol as a substrate(Received February 1, 1991, Accepted March 25, 1991).

Polyphenol oxidase(o-diphenol : $O_2$ oxidoreductase, EC 1.10.3.1) is a copper-containing enzyme which catalyzes either one or two reactions involving molecular oxygen. The first type of reaction is hydroxylation of monophenols leading to formation of o-dihydroxy compounds. The second type of reaction is oxidation of o-dihydroxy compounds to quinones[1].

Polyphenol oxidase is also known as phenol oxidase, tyrosinase, o-diphenol oxidase, catechol oxidase, phenolase and chlorogenic acid oxidase.

Brown discoloration of edible mountain herb and its concentrate was found to be related to the enzymatic browning that takes place before or during processing[2,3].

We previously reported some properties of polyphenol oxidase from *Ligularia fischeri* and *Aster scaber*[4,5].

Since polyphenol oxidase in *Spuriopimpinella bracycarpa* has not been adequately investigated, the purpose of this investigation was to purify the polyphenol oxidase complex of *Spuriopimpinella bracycarpa* and its some properties and specificity.

## Materials and Methods

### Materials

Edible mountain herb, *Spuriopimpinella bracycarpa* were purchased on a local market. Particulate fractions studied were generally obtained in the following manner : *Spuriopimpinella bracycarpa* were cut into slices, rinsed with tap water and moistened with acetone and then chilled to 4℃.

For the extraction, the mixtures were homogenized in a Waring blender for 2min with McIlvaine buffer solution (pH 6.0). The homogenates were filtered and treated with cold acetone, after that, white acetone powder sample was obtained from the homogenates.

### Protein determination

The protein in effluents of gel filtration was estimated from absorbance at 280nm, assuming that the absorbance at 280nm at the concentration of 1mg per ml is 1.0. The protein concentration in polyphenol oxidase solution was determined from absorbance at 750nm by the method of Lowry[6], using bovine serum albumin as the standard.

Key words : Polyphenol oxidase, *Spuriopimpinella bracycarpa*
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**Measurement of enzyme activity and enzyme unit**

Enzyme activity was determined by measuring the rate of increase in absorbance at 420nm at 25℃ in a model of CECIL universal automatic scanning spectrophotometer. The reaction mixture contained 2ml of 0.1M catechol solution, 1ml of distilled water and 20$\mu$l of the enzyme solution. The reference cuvette contained only substrate solution and distilled water. One unit of polyphenol oxidase activity is defined as the amount of enzyme that causes a 0.01 extinction change in absorbance at 420nm per min at 25℃[7].

**Purification of enzyme**

All the procedures were carried out in a chromatochamber at about 4℃. After addition of powdered $(NH_4)_2SO_4$ to 80% saturation, the precipitate was collected, dissolved in 20ml of McIlvaine buffer solution (pH 6.0), and dialysed for 12hrs against water, and the remaining undissolved precipitate removed by centrifugation. The resulting solution(2ml) of clear supernatant(30ml) was immediately applied, without further concentration, to a column of Sephadex G-150. A column(2.5×40cm) filled with Sephadex G-150 was conditioned with a 0.02M phosphate buffer solution(pH 6.0) containing 0.1M sodium chloride. The enzyme solution (2ml) was then applied to the column and eluted with the same buffer solution at the flow rate of 10ml per hr.

**Enzyme preparation**

1g of frozen acetone powder(−20℃) sample were homogenized in 80ml of McIlvaine buffer solution(pH 6.0). The homogenate was centrifuged at 10,000g for 30min. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 80% saturation. The solution was stirred for 30min, and centrifuged precipitate recovered and dissolved in 0.02M phosphate buffer solution(pH 6.0) and dialyzed against the same buffer solution overnight.

**Chemicals**

Sephadex G-150 was purchased from Pharmacia Fine Chemicals(Sweden). Column was purchased from Fuji Kagaku Kogyo Co., Ltd.(Toyama, Japan). Other chemicals were the best commercial products available.

## Results and Discussions

**Purification of enzyme**

Three polyphenol oxidase were isolated from the crude extract of a *Spuriopimpinella bracycarpa* by gel filtration on Sephadex G-150(Fig. 1). Polyphenol oxidase II was

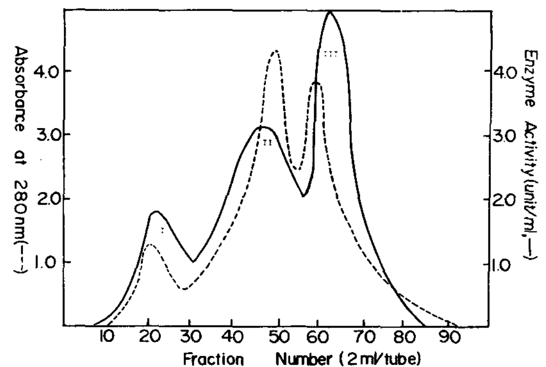


Fig. 1. Gel filtration of polyphenol oxidase from *Spuriopimpinella bracycarpa* on a Sephadex G-150 column

Table 1. Summary of the purification of polyphenol oxidase from *Spuriopimpinella bracycarpa*

| Purification steps | Volume (ml) | Activity (units/ml) | Total activity (units) | Protein (mg/ml) | Specific activity (units/mg) | Activity recovery | Fold of purification |
|---|---|---|---|---|---|---|---|
| Crude extract | 20.00 | 90.00 | 1,800.00 | 102.85 | 0.87 | 100.00 | 1.00 |
| $(NH_4)_2SO_4$ precipitate | 8.00 | 112.00 | 896.00 | 109.35 | 1.02 | 49.77 | 1.17 |
| Chromatography on Sephadex G-150 | | | | | | | |
| I | 28.00 | 1.63 | 45.64 | 0.95 | 1.71 | 2.53 | 1.96 |
| II | 40.00 | 2.65 | 106.00 | 2.76 | 0.96 | 5.88 | 1.10 |
| III | 20.00 | 2.84 | 56.80 | 2.21 | 1.28 | 3.15 | 1.47 |

the main component and accounted for 50.8% of the total activity of the three polyphenol oxidases. Our previous papers[4,5] have reported that polyphenol oxidases from *Ligularia fischeri* and *Aster scaber*. Four and five polyphenol oxidases were partially isolated from the crude extract of a *Ligularia fischeri* and *Aster scaber* by Sephadex G-150 chromatography.

Table 1 summarized the increases in the specific activity of polyphenol oxidase and the yield during the purification. After the $(NH_4)_2SO_4$ treatment, a purification fold of 1.17 was obtained and the specific activity was 1.02 units/mg. Polyohenol oxidase II, which contained 106.0 units of polyphenol oxidase activity.

**Effect of pH and temperature on the activity of the purified polyphenol oxidase**

The polyphenol oxidase activity was measured at various pHs from 5.0 to 8.5, using a 0.1M citric acid-0.2M phosphate buffer solution. The optimum pH for the activity was found to be arround 7.5(Fig. 2).

The enzyme activity was measured at various temperature, from 20℃ to 50℃. The optimum temperature for the activity was arround 30℃(Fig. 3).

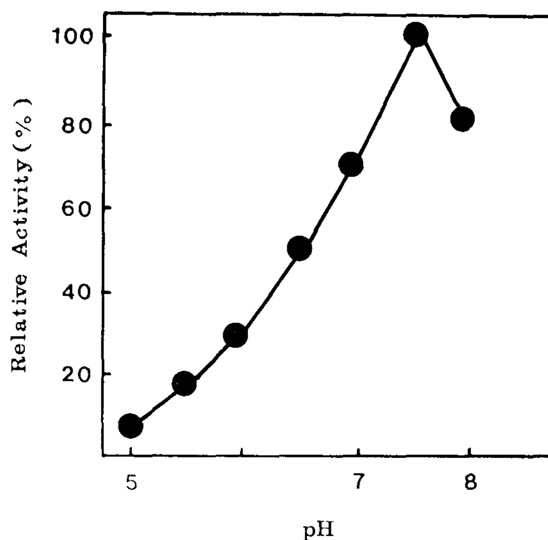


Fig. 2. Effect of pH on polyphenol oxidase activity of *Spuriopimpinella bracycarpa*.

The polyphenol oxidase activities were determined in 0.1M citric acid-0.2M phosphate buffer ranging from pH 5.0 to 8.0.

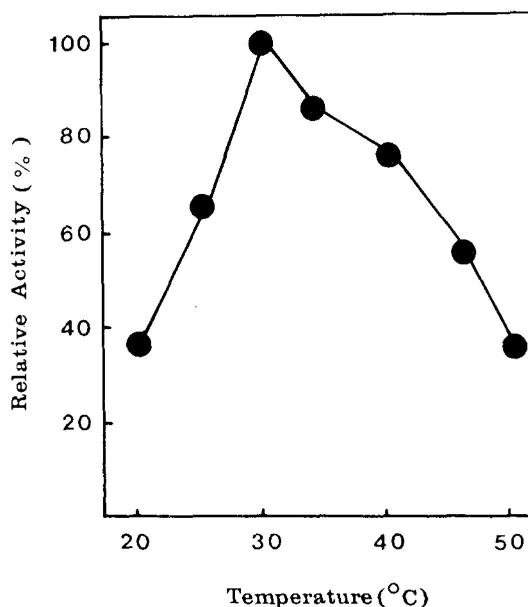


Fig. 3. Effect on temperature on polyphenol oxidase activity of *Spuriopimpinella bracycarpa*.

The polyphenol oxidase activity was determined at various temperature between 20℃ and 50℃ for 10min.

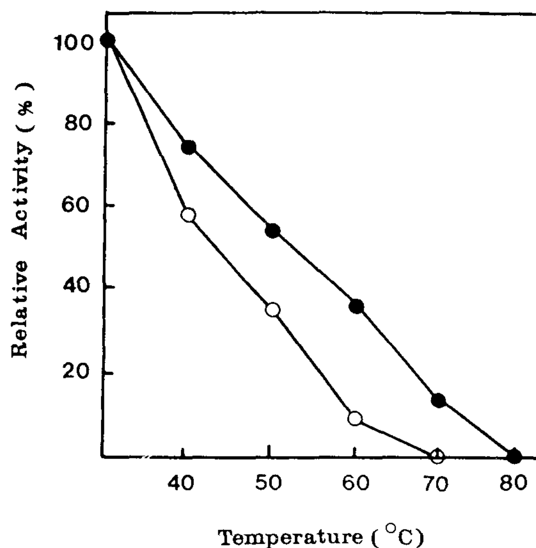


Fig. 4. Thermal stability of *Spuriopimpinella bracycarpa* polyphenol oxidase.

The enzyme solutions were heated at various temperarure(40~80℃) for 5 and 30min. After heating, remaining enzyme activities were determined with catechol as substrate at pH 6.5 and 35℃.

● : 5min, ○ : 30min.

**Effects of temperature on the stability of the purified polyphenol oxidase**

The enzyme treated in a water bath at various temperatures from 40℃ to 80℃ for 5 and 30min at pH 6.5 in the 0.1M citric acid-0.2M phosphate buffer solution, and then remaining activity was determined with catechol as a substrate. The enzyme was completely inactivated when examined at 70℃ for 30min and at 80℃ for 5min at pH 6.5(Fig. 4). In comparison with the values reported for the polyphenol oxidase from *Ligularia fischeria* and *Aster scaber*, the values of these enzymes were similar to one another, though small difference were found among them.

**Effects of various compounds on the activity of the purified polyphenol oxidase**

The effects of various compounds(0.05, 0.1, 0.5 and 1mM) on the activity of the purified polyphenol oxidase shown in Table 2. The enzyme was inhibited by treatment with ascorbic acid, glutathione and pota-ssium cyanide(0.1mM). However the enzyme was completely inhibited by L-cysteine, ascorbic acid, glutathione and potassium cyanide(0.5 and 1mM). In contrast with other compounds, the enzyme activity was not inhibited considerably by treatment with high concentration(0.5 and 1mM) of EDTA.

Table 2. Effect of various inhibitors on *Spuriopimpinella bracycarpa* polyphenol oxidase activity

Relative activity(%)

| Inhibitor(mM) | Concentration(mM) | | | |
|---|---|---|---|---|
| | 0.05 | 0.1 | 0.5 | 1 |
| None | 100 | 100 | 100 | 100 |
| L-Cysteine | 89 | 93 | 0 | 0 |
| Ascorbic acid | 94 | 50 | 0 | 0 |
| Glutathione | 78 | 53 | 0 | 0 |
| Potassium cyanide | 63 | 50 | 0 | 0 |
| EDTA | 78 | 75 | 72 | 70 |

On the other hand, *Ligularia fischeri* enzyme was strongly inactivated by ascorbic acid and potassium cyanide(0.5mM). And completely inhibited by L-lysine, glutathione(0.5 and 1mM), ascorbic acid and potassium cyanide(1mM).

**Substrate specificity and enzyme kinetics.**

The purified polyphenol oxidase showed good activity on catechol and 3, 4-dihydroxytoluene but was strongly inactivated on pyrogallol, dopamine and DL-dopa(Table 3) The effect of the concentration of catechol on activity was examined as shown in Fig. 5. The apparent $K_m$ for this substrate was about 86.5mM. In comparison with the substrate specificity and $K_m$ value of the enzyme from

Table 3. Substrate specificity of *Spuriopimpinella bracycarpa* polyphenol oxidase

| Substrate(10mM) | Relative activity(%) |
|---|---|
| Catechol | 100.0 |
| Chlorogenic acid | 95.0 |
| 3, 4-Dihydroxytoluene | 100.0 |
| Pyrogallol | 17.5 |
| Hydroxyhydroquinone | 62.5 |
| Dopamine | 15.0 |
| DL-Dopa | 7.5 |

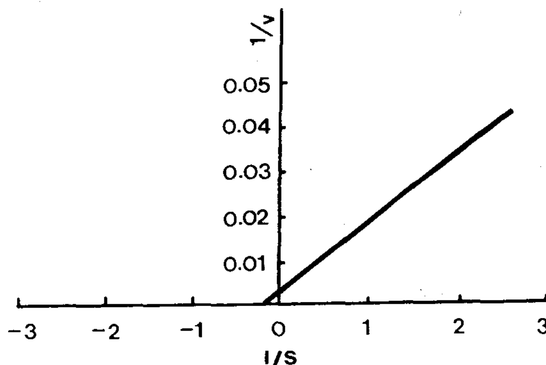


Fig. 5. Lineweaver-Burk plot of *Spuriopimpinella bracycarpa* polyphenol oxidase activity.

Concentration of catechol ranging fron $2\times10^{-2}$M to $4\times10^{-3}$M in 0.1M citic acid-0.2M phosphate buffer pH 7.5 were used for this study.

*Ligularia fischeri* and *Aster scaber*, *Ligularia fischeri* enzyme has good activity on catechol and chlorogenic acid but was inactive on dopamine. And also, *Aster scaber* enzyme has good activity on chlorogenic acidbut was inactive on DL-dopa. And the $K_m$ value of *Aster scaber* enzyme is close to that of *Ligularia fischeri* enzyme.

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## 참나물로부터 추출한 polyphenol oxidase의 부분정제 및 성질

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**초록** : 참나물로부터 추출한 polyphenol oxidase의 부분정제를 $(NH_4)_2SO_4$ 처리 및 Sephadex G-150 column chromatography에 의해 분리하였다. Polyphenol oxidase의 최적 pH와 최적온도는 7.5와 30℃였으며, pH 6.5에서 70℃에서 30분간 처리시와 80℃에서 5분간 처리시 완전 실활하였다. Polyphenol oxidase는 ascorbic acid, glutathione, potassium cyanide(0.1mM)에 의해 불활성화되었으며 L-cysteine, potassium cyanide, ascorbic acid, glutathione(0.5, 1mM)에 의해서는 완전 실활되었다. Catechol과 3, 4-dihydroxytoluene의 기질은 높은 특이성을 나타낸 반면 pyrogallol, dopamine, DL-dopa의 기질은 강하게 활성을 억제하였다. Polyphenol oxidase의 $K_m$ 값은 86.5mM이었다.