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Purification and Some Properties of the Polyphenol Oxidase from Ascidian, *Halocynthia roretzi*

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Abstract

Polyphenol oxidase(PPO) isolated from the crude extract of ascidian, *Halocynthia roretzi*, showed higher affinity for catechol than tyrosine or DL-DOPA. Successful enzyme assay could be performed at 25°C, 10 min. by mixing 0.2ml of crude enzyme extract with 2.8ml of 0.13M catechol in 0.1M sodium phosphate buffer (pH 6.4). The specific activity of PPO which had been purified with a combination of ammonium sulfate treatment, ion exchange chromatography on DEAE-cellulose, and gel filtration on Sepharose 6B was 13-fold higher than that of crude extract. The purified enzyme was homogeneous as confirmed by polyacrylamide disc gel electrophoresis. The activity of PPO was stable from pH 5.0 to 8.0 and showed the peak activity at pH 6.4. The optimum reaction temperature for PPO oxidation on catechol was 35°C and those enzyme were heat stable up to 40°C. Molecular weight of the enzyme was estimated about 170 kDa. One molecule was found to be composed of four subunits. Two of them had molecular weight of 55 kDa and the others 30 kDa. The K_m values, V_{max} and catalytic efficiency(V_{max}/K_m) for catechol were 0.12mM, 2.56mM/liter/min. and 0.18min^{-1} respectively. The substrate affinity and electrophorectic pattern suggested that the enzyme of ascidian was considered to be not tyrosine but catechol oxidase.

Key words: ascidian, polyphenol oxidase, purification, enzymatic property

INTRODUCTION

Unfavorable enzymatic browning occurred in many plants and seafood products has been a great concern to food technologists and processors. This discoloration is not a chemical quality defect ; however, it is not appealing to consumers and thus reduces the market value. Enzymatic browning caused by polyphenol oxidase (PPO) has been extensively studied in fruits and vegetables(1-5) but it has received a little attention in economically important crustacean species such as crab, shrimp, and lobster recently(6-12). But the research regarding this activation phenomena cannot be found in ascidian among seafood products.

The ascidian, *Halocynthia roretzi*, has been esteemed as a special seafood dish in Korea because of its specific flavors and tastes. This ascidian is well known for its discoloration during storage of processed products such as salt-fermented(13,14) and vegetable oil packed products(15). The dark discoloration of those products has been considered to be caused by the reaction of PPO(1-3). However, a better understanding of the properties of this enzyme would facilitate development of treatments/manipulations to control the postmortem dark discoloration phenomenon. In the present study, PPO isolated from live ascidian was characterized with respect to their response to substrate, pH and temperature, as well as kinetic and thermodynamic properties.

MATERIALS AND METHODS

Materials

Fresh ascidians, *Halocynthia roretzi*, was obtained from a local seafood market and directly transported in ice to laboratory. Chopped muscles were a source of extraction and purification of PPO. DEAE-cellulose, Sepharose 6B, mark proteins and reagents of electrophoresis used in this study were purchased from Sigma Chemical Co.

Crude enzyme solution preparation

Crude PPO was extracted according to the procedure

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of Ingebrigtsen et al.(16) with a slight modification. Ascidian meats were homogenized in 10 volumes of 0.1M sodium phosphate buffer and centrifuged at 8,600×g at 4°C for 30min to remove insoluble materials and the supernatant was stored at -20°C until use.

Experimental conditions for enzyme activity

Tyrosine, catechol and DOPA were used for substrate affinity assay. The assay mixture contained 0.1M sodium phosphate buffer(pH 6.4), substrate(0.01~0.2M) and crude enzyme in a volume of 3ml. One unit of enzyme acivity was defined as change of 0.001 absorbance per min at 25°C. The effect of reaction time on enzyme activity was studied by measuring the highest enzyme activity of crude enzyme on the preincubated (5min at 25°C) mixture of substrate(catechol) and buffer. The optimum temperature of PPO-catechol reaction was determined by adding 0.2ml of crude enzyme in the pre-equilibrated mixture of 0.05M catechol in 2.8ml of 0.1M sodium phophate buffer. Equilibration was performed for 5min. at temperature from 15 to 65°C. The optimum pH of crude enzyme was determined by preparing the substrate(0.05M catechol) in various buffer solutions and adding 0.2ml of crude enzyme to 2.8ml of the buffered substrate. Composition of the buffer solutions were ; 0.1M glycine- HCl, pH 3.0~4.0 ; 0.1M sodium acetate-acetic acid, pH 4.0~6.0 ; 0.1M NaH_2PO_4-, Na_2HPO_4, pH 6.0~8.0 ; 0.1M tris-HCl, pH 8.0~9.0 ; 0.1M glycine-NaOH, pH 9.0~10.0.

Extraction and purification of PPO

Eighty-grams of fresh ascidian meat was homogenized with 120ml of 0.1M sodium phosphate buffer(pH 6.4) at 4°C for 2min. Homogenate was centrifuged at 8,600×g at 4°C for 30min, and the supernatant was stored at -20°C until use. The supernatant was fractionated with ammonium sulfate, and the precipitate between 30% and 80% saturation was collected by centrifugation at 2,000×g at 4°C for 15 min. This was redissolved in 50ml of 0.1M sodium phosphate buffer (pH 6.4) and dialyzed against three changes of extraction buffer(10mM sodium phosphate buffer, pH 6.4, 3L). The dialyzed extract was loaded onto a DEAE-cellulose column(2.5×40cm) which had been pre-equilibrated with 10mM sodium phosphate buffer(pH 6.4). After loading of sample, the system was programmed to elute using a gradient of 0.2~1.3M KCl in 600ml of the same buffer at the flow rate of 60ml/h. The collected solutions were applied to a Sepharose 6B column(2.1×36cm) equilibrated with 0.1M sodium phosphate buffer(pH 6.4). The elution was performed with an upward flow of the same buffer and 33ml of active enzyme fractions was collected at a flow rate of 20ml/h.

Protein quantitation and molecular weight determination

PPO activity was assayed spectrophotometrically using catechol as a substrate. The reaction mixture comprised 0.8ml of 0.13M catechol and 2.0ml of 0.1M sodium phosphate buffer(pH 6.4). Partially purified enzyme extract 0.2ml was added to the mixture which had been pre-equilibrated at 31°C for 5min. prior to enzyme addition. PPO activity was monitored by observing the increase in absorbance at 410nm for 10 min. Protein concentration was determined by the method of Lowry et al.(17) using bovine serum albumin as standard. Sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE) according to the modified procedure described by Laemmli(18) was used for molecular weight determination of the enzyme. The disc-gel system was equipped with 12 sample columns and each column was consisted of a 6-cm separating gel(7.5% polyacrylamide) and a 1-cm stacking gel(3% polyacryl amide). The partially purified enzyme extract and standard proteins were reduced by 2-mercaptoethanol for 5min and 20μl of each extract loaded on column. 25mM tris-192mM glycine buffer solution(pH 8.3) containing 0.1% SDS was used as the electrode buffer, 2 or 3 drops of 0.5% bromophenol-blue were added in upper part electrode buffer. The disc-gel was run at a constant current of 1.5mA per column when protein band existed in stacking gel but 3mA applied separating gel. After electrophoresis, protein bands were stained with dying solution containing 0.25% coomassie brillinat blue(R-250) in ethanol, acetic acid and water mixture (9 : 2 : 9, v/v/v) for 5 hours. Stained gel was soaked in destaining solution(ethanol, acetic acid and water, 25 : 8 : 65, v/v/v), and renewed destaining solution every 1 hour to complete destaining. Protein standards used to estimate molecular weight were ; carbonic anhydrase (29 kDa), egg albumin(45 kDa), bovine serum albumin (66 kDa), phosphorylase b(97.4 kDa), β-galactosidase (116 kDa), and myosin(205 kDa).

Optimum temperature and pH on stability

To determine temperature stability on PPO activity, the enzyme extract was incubated at temperatures from 10 to 70°C for 30min. Following equilibration in an ice bath for 5min, enzyme extract(0.2ml) was mixed with 2.8ml of 0.05M catechol in 0.1M sodium phosphate buffer(pH 6.4) and residual enzyme activity was measured as relative activity(19). For influence of pH on the activity of PPO, 0.2ml of the enzyme extract was incubated in 0.4ml of various buffer solutions ranging from pH 3.0 to 10 at 31°C for 30min. 0.3ml of preincubated PPO extracts was mixed with 2.7ml of 0.05M catechol in 0.1M sodium phosphate buffer(pH 6.4) and residual PPO activity was checked as the relative activity(19) spectrophotometrically.

Enzyme kinetics

Kinetic parameters(K_m and V_{max}) of PPO from ascidian were determined using the Lineweaver and Burk equation(20). Catechol solutions at concentrations from 2 to 25mM in 0.1M sodium phosphate buffer(pH 6.4) were used as substrate and all assays were done at ambient conditions.

RESULTS AND DISCUSSION

Optimal reaction condition of crude PPO extract

To find out substrate affinity of crude PPO extract from ascidian meat, tyrosine, catechol and DOPA were used as substrates. As shown in Fig. 1, crude PPO extract could have 3-fold higher substrate affinity(17.5 units/ml/min) for catechol than for tyrosine(6.5units/ml/min) or DOPA(7.5units/ml/min). With respect to substrate-related activity, our results indicated that latent enzyme activity of crude PPO extracts was associated with catechol but not with tyrosine or DOPA hydroxylase activity. The observation of substrate affinity was similar to the findings made by Oshima and Nagayama(21). Thus, catechol was used as a substrate of PPO from ascidian meat in following enzyme assays. PPO activity increased gradually with increasing concentration of catechol up to 0.13M in sodium phosphate buffer(pH 6.4). 3ml of total reaction volume was composed of 0.2ml of crude PPO extracts and 2.8ml of those catechol solution. It was also observed that activity of crude PPO extracts increased with the time of reaction and those activity reached a maximum in 10min at the same conditions as described above.

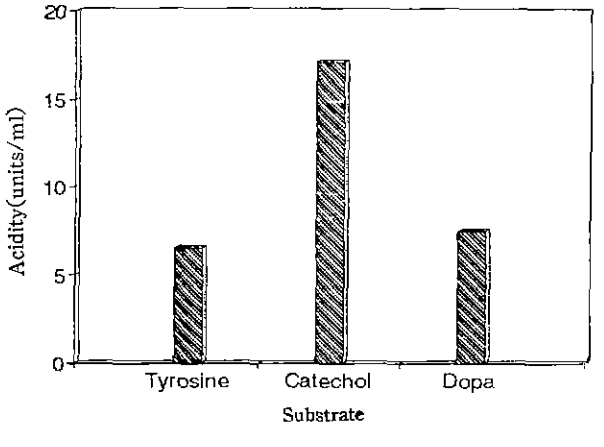

Fig. 1. Substrate affinity of ascidian PPO with tyrosine, catechol and DL-DOPA.
Enzyme reaction was performed at 25°C for 10min using enzyme-substrate comprised 0.2ml of crude enzyme solutions and 2.8ml of 0.05M substrates in 0.1M sodium phosphate buffer(pH 6.4).

The crude PPO extract showed the temperature-dependent enzyme activity which risen with temperature between 10°C and 30°C, and those activity dropped instantly at temperatures higher than 30°C(Fig. 2). The optimum temperature of crude PPO for catechol oxidation was not similar to values reported for PPOs of other seafood resources. For example, the optimum temperature for the oxidation of catechol by antarctic krill PPO's was 25°C(21) and the optimum temperature for lobster PPO-catechol reaction was 45°C(22).

The effect of pH on the decomposition of catechol by crude PPO is presented in Fig. 3. The data showed that crude PPO of ascidian was the most active at pH 6.5, and the lower(pH 4) or higher(pH 10) pH resulted

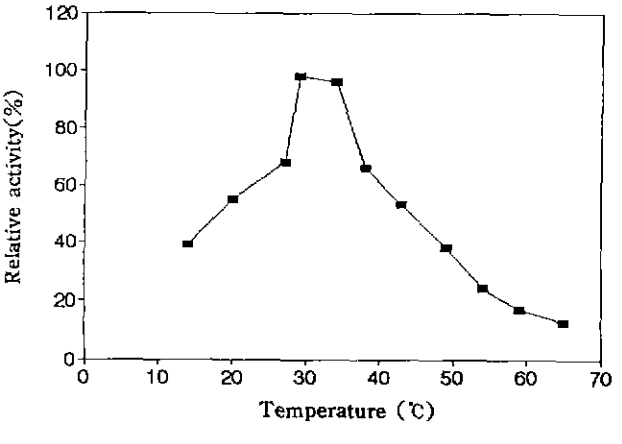

Fig. 2. Temperature dependence on the oxidation of the catechol by crude ascidian PPO extract.

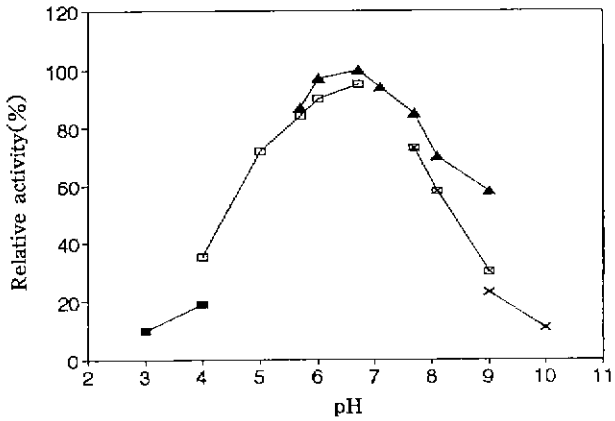

Fig. 3. pH dependence on the oxidation of catechol by crude ascidian PPO extract.
■ : Glycine-HCl, ⊟ : Sodium acetate-acetic acid
▲ : Na_2HPO_4-NaH_2PO_4, ⊠ : Tris-HCl
✕ : Glycine-NaOH

in 20% or 10% of relative activities. The pH-activity profiles of crude ascidian PPO was similar to those reported for PPO from marine crustacean species; e.g., optimum pH 6.2~8.0 for lobster(22) and 6.5 for krill(21). In case of DOPA as the substrate, PPO from crustacean had a broad optimal pH range(6.5~9.0 for pink shrimp, 6~8 for lobster)(6,9).

Purification of ascidian PPO

The results of purification for ascidian PPO are summarized in Table 1. From 80 grams of homogenated ascidian meat with 120ml of 0.1M sodium phosphate butter(pH 6.4), 175ml of supernatant(crude enzyme extract) could be obtained after centrifugation at 8,600× g at 4°C for 30min. Ammonium sulfate fractions were 150ml for 30% saturation and 62ml for 80% saturation. When the 86ml of dialyzed enzyme solution eluted on DEAE-cellulose column using a linear gradient of 0.2 to 1.3M KCl in 600ml same buffer as above at a flow rate of 60ml/h, 60ml of enzyme soultion was collected from active fractions which could be indentified in the range from fraction 29 to 40. Purified enzyme solution was obtained when the enzyme solution from DEAE-cellulose was further fractionated by Sepharose 6B column Chromatography. The specific activity of PPO fraction after gel filtration on Sepharose 6B was approximately 13 times higher than that of the crude homogenate and recovery against the crude enzyme preparation was 30%.

Electrophoresis and molecular weight determination

Fig. 4 shows the electrophoresis patterns of ascidian PPO at each stage of purification. The ascidian PPO fraction after ion-exchange chromatography migrated as one major band, and two bands in the presence of SDS. The molecular weights of ascidian PPO were determined to be 170, 55, and 30kDa. The molecular weight of 170kDa was thought to be as major ascidian PPO having higher molecular weight than those of other seafood PPOs(shrimp, 30~40kDa; antarctic krill 75 and 83kDa)(6,21,23), but lower than that of brown shrimp(210kDa)(24). The presence of two band(molecular weight, 55 and 30kDa) suggested that ascidian PPO had two subunits, and similar to the findings by other investigators(25).

Temperature and pH stability

The influence of temperature on the stability of ascidian PPO is presented in Fig. 5. The PPO from ascidian was stable up to 40°C but heat labile at higher temperatures. For instance, it retained only about 10% of its original activity after 30min. incubation at 70°C. In this respect, heat stability of ascidian PPO with catechol differed from those of lobster(8) and krill(26) PPOs, which retained more than 90% of the original activity after 1 hour incubation at 30°C. The temperature stability of ascidian PPO of catechol oxidation was similar

Table 1. Purification scheme for ascidian polyphenol oxidase

Purification step	Volume(ml)	Activity	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg)	Activity recovery
Crude extract	175	23	4025	0.89	25.8(1.00)	100.0
30% salting out supernatant	150	30	4500	0.69	43.5(1.69)	111.8
80% salting out precipitate	62	51	3162	0.72	70.8(2.74)	78.6
Dialysis	86	43	3698	0.75	57.3(2.22)	91.9
DEAE-cellulose elute	60	53	3180	0 28	189.3(7.33)	79.0
Sepharose 6B elute	33	37	1221	0.11	336.4(13.04)	30.3

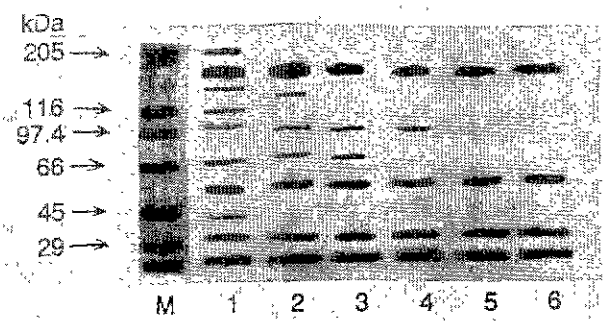

Fig. 4. SDS-PAGE patterns of ascidian PPO at various stages of purification.
Lane M: molecular weight markers, Carbonic anhydrase(29kDa), egg albumin(45kDa), bovine serum albumin(66kDa), phosphorylase b(97.4kDa), β-galactosidase(116kDa), myosin(205kDa), Lane 1: crude extract, Lane 2: 30% salting out supernatant, Lane 3: 80% salting out precipitate, Lane 4: after dialysis, Lane 5: DEAE-cellulose fraction, Lane 6: Sepharose 6B fractions.

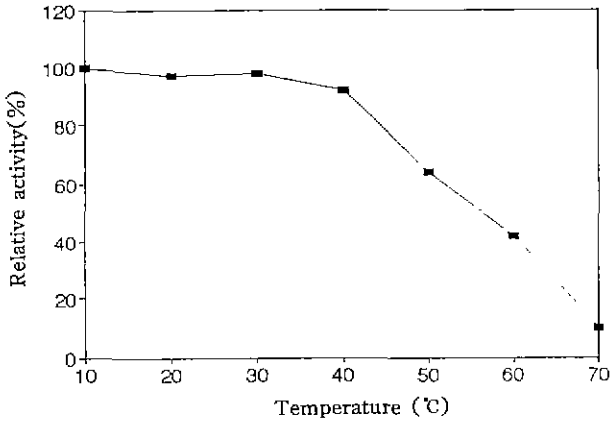

Fig. 5. Stability of purified ascidian PPO on the oxidation of the catechol at various preincubation temperature.
The enzyme was preincubated for 30min at each temperature.

to that of Florida spiny lobster which was rapidly inactivated after 30min. at a temperature of above 40°C(7).

The pH stability curve indicated that ascidian PPO was most stable at a weak acidic pH. Ascidian PPO reatined more than 90% of its initial activity at a pH range from 5.5 to 7.5(Fig. 6). Those results were consistent with the findings by Ferrer et al.(7) who reported that activated Florida spiny lobster PPO was most stable over a broad pH range of 6 to 9, while shrimp PPO was found to be relatively stable from 7 to 9 on DOPA as a substrate.

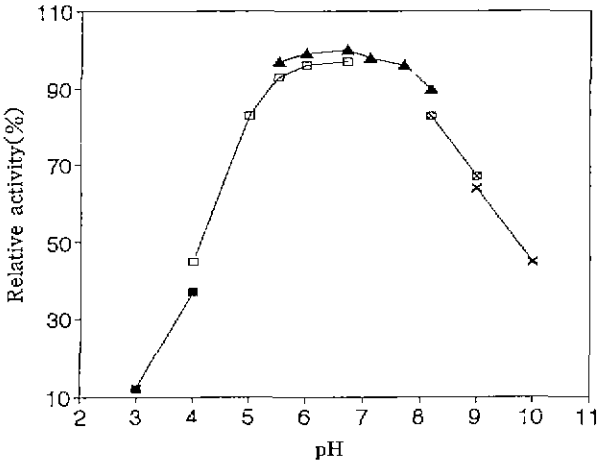

Fig. 6. pH stability of purified ascidian PPO on the oxidation of the catechol after preincubated 30min at 31°C.
■ : Glycine-HCl, ⊟ : Sodium acetate-acetatic acid
▲ : Na_2HPO_4-NaH_2PO_4, ⊞ : Tris-HCl
✕ : Glycine-NaOH

Table 2. Kinetic parameters of the ascidian PPO against catechol substrate

Parameters	Values
K_m(mM)	0.12
V_{max}(mmol/L/min)	2.56
V_{max}/K_m(min^{-1})[1)]	21.30

[1)]Physiologic efficiency

Kinetic parameters of ascidian PPO

The kinetic properties of ascidian PPO is summarized in Table 2. Ascidian PPO displayed a relatively lower Michaelis contant(K_m=0.12mM) and a greater maximum velocity(V_{max}=2.56mM/liter/min) than for the same catechol oxidation by lobster(K_m=4.58, V_{max}= 0.72mM/liter/min)(27). Using DL-DOPA as a substrate, Simpson et al.(6) showed pink shrimp PPO had a relatively lower K_m of 1.6mM. Summers(28) also reported the fiddler crab's blood PPO had a K_m value of 0.50mM. The catalytic efficiency, defined as the ratio V_{max}/K_m, was estimated to be 2.13. Thus, based on the catalytic efficiencies, ascidian PPO exhibited a higher affinity for catechol than those of lobster PPO for catechol (0.013 and 0.157)(9).

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