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Purification and Characterization of Polyphenol Oxidase from Garland Chrysanthemum (*Chrysanthemum coronarium* L.)

ELINE NKYA, CHIE KOUNO, YING-JIE LI, CHANG-PENG YANG, NOBUYUKI HAYASHI, AND SHUJI FUJITA*

Laboratory of Food Science, Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Saga 840-8502, Japan

Polyphenol oxidase (PPO) of garland chrysanthemum (*Chrysanthemum coronarium* L.) was purified ~32-fold with a recovery rate of 16% by ammonium sulfate fractionation, ion exchange chromatography, hydrophobic chromatography, and gel filtration. The purified enzyme appeared as a single band on PAGE and SDS-PAGE. The molecular weight of the enzyme was estimated to be about 47000 and 45000 by gel filtration and SDS-PAGE, respectively. The purified enzyme quickly oxidized chlorogenic acid and (−)-epicatechin. The K_m value (Michaelis constant) of the enzyme was 2.0 mM for chlorogenic acid (pH 4.0, 30 °C) and 10.0 mM for (−)-epicatechin (pH 8.0, 40 °C). The optimum pH was 4.0 for chlorogenic acid oxidase (ChO) and 8.0 for (−)-epicatechin oxidase (EpO). In the pH range from 5 to 11, their activities were quite stable at 5 °C for 22 h. The optimum temperatures of ChO and EpO activities were 30 and 40 °C, respectively. Both activities were stable at up to 50 °C after heat treatment for 30 min. The purified enzyme was strongly inhibited by L-ascorbic acid and L-cysteine at 1 mM.

KEYWORDS: Polyphenol oxidase; garland chrysanthemum; chlorogenic oxidase activity; (−)-epicatechin oxidase activity; purification

INTRODUCTION

Garland chrysanthemum (*Chrysanthemum coronarium* L.) is a popular vegetable in Japan. During processing of this vegetable, undesirable browning occurs in the damaged tissue, mainly due to oxidation of polyphenols by polyphenol oxidese (EC 1.10.3.1; *o*-diphenol:oxygen oxidoreductase, PPO). In this plant and edible burdock, browning has been known to be mainly caused by enzymatic oxidation of the chrologenic acid derivatives (*1*). Enzymatic browning is also found in many fruits and vegetables, resulting in lowered marketability. To prevent such browning, many investigations have been conducted to characterize PPOs of many fruits and vegetables (*2*−*13*), particularly on substrate specificities for such polyphenols as pyrocatechol, 4-methylcatechol, DL-dopa, dopamine, (−)-epicatechin, pyrogallol, and phloroglucinol (*2*−*6*, *12*−*19*). In our laboratory, we isolated and characterized PPOs, which mainly oxidize chlorogenic acid, from eggplant (*7*) and head lettuce (*8*). The PPO from eggplant oxidized chlorogenic acid more strongly than did PPO from head lettuce, which belongs to the family of garland chrysanthemum. Although the PPO from garland chrysanthemum had strong oxidative activity on chlorogenic acid and (−)-epicatechin, the characteristics of this enzyme had not been investigated in detail so far. In the present study, garland chrysanthemum PPO was purified using chlorogenic acid as substrate, and the properties of the purified enzyme were investigated.

MATERIALS AND METHODS

Materials. Garland chrysanthemum was purchased at local markets in Saga, Japan. DEAE-Cellulofine was purchased from Chisso, Tokyo, Japan. Butyl-Toyopearl 650M and Toyopearl HW 55-Superfine (HW55-S) were obtained from Toso Co., Tokyo, Japan. CM-Sephadex C-50 was obtained from Pharmacia Co., Uppsala, Sweden. The other reagents used were purchased from Wako Pure Chemical Co., Osaka, Japan, and Katayama Chemical Co., Osaka, Japan.

Assay of Enzyme Activity. *PPO Activity.* PPO activity was measured according to the colorimetric method (*6, 7, 18*) for the reaction mixture containing 0.5 mL of 10 mM aqueous solution of various polyphenols (see **Table 2**), 4 mL of 0.1 M citrate/0.2 M sodium phosphate buffer (McIlvaine buffer, pH 4), and 0.5 mL of enzyme solution. After 5 min of incubation of the mixture at 30 °C, the increase in absorbance at 420 nm (ΔA_{420}) was measured using a Shimadzu MPS-2000 spectrophotometer. One unit of the enzyme activity was defined as an increase of absorbance at 420 nm of 0.1 per minute and per milliliter of enzyme solution (1 cm light path).

Chlorogenic Acid Oxidase (ChO) Activity. The spectrophotometric method developed by Tono et al. (*20*) was employed to measure ChO activity. The mixture to be tested consisted of 0.5 mL of 0.4 mM chlorogenic acid, 1 mL of McIlvaine buffer (pH 4), and 0.5 mL of the enzyme solution and was incubated at 30 °C for 5 min. After incubation, the reaction was stopped by the addition of 3 mL of 2% metaphosphoric acid solution. For the control, 0.5 mL of the enzyme solution was added to a mixture of 0.5 mL of 0.4 mM chlorogenic acid solution, 1 mL of

* Author to whom correspondence should be addressed (fax +81-952-28-8709; e-mail fujitas@cc.saga-u.ac.jp).

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McIlvaine buffer (pH 4), and 3 mL of 2% metaphosphoric acid solution. The difference in absorbance at 325 nm (ΔA_{325}) between the control and test solution was measured by a Shimadzu MPS 2000 spectrophotometer. One unit of the enzyme activity was expressed as a decrease in absorbance at 325 nm (ΔA_{325}) of 0.01 per minute per milliliter of the enzyme solution (1 cm light path).

Protein Determination. Protein was determined according to the method of Hartree (*21*) using a bovine serum albumin (fraction V, Katayama Chemical Co., Osaka, Japan) as standard. In the chromatography, protein was determined by measuring the absorbance at 280 nm.

Enzyme Purification. All steps of purification were carried out at 4 °C. The whole plant of garland chrysanthemum (fresh weight = 3.0 kg) was homogenized with 1500 mL of 0.1 M potassium phosphate/0.1 M sodium phosphate buffer (0.1 M phosphate buffer, PB, pH 7) containing 1% L-ascorbic acid. After filtration of the homogenate through cotton cloth, the filtrate was centrifuged at 10300*g* for 20 min, and the supernatant was brought to 80% ammonium sulfate saturation. After stirring overnight, the protein precipitate was collected by centrifugation at 10300*g* for 20 min, dissolved in a small volume of 0.01 M PB (pH 7), and then dialyzed against the same buffer for 36 h with the change of dialyzed media four or more times. The dialyzed solution (crude enzyme) was applied to a DEAE-Cellulofine AL column (4 × 14.5 cm) equilibrated with 0.01 M PB (pH 7), and then the enzyme fractions that were not adsorbed on the column were collected. The collected PPO fractions were applied to a CM-Sephadex C-50 column (3 × 15 cm) equilibrated with 0.01 M PB (pH 7) and eluted with the same buffer. The enzyme fractions were collected and brought to contain 1 M ammonium sulfate and then applied to a butyl-Toyopearl column (1.6 × 11 cm) equilibrated with 0.01 M PB containing 1 M ammonium sulfate and eluted with a linear gradient of ammonium sulfate (from 1 to 0 M in 0.01 M PB, pH 7). The enzyme active fractions were pooled and dialyzed against 0.01 M phosphate buffer with the change of dialyzed media four times or more. The dialyzed enzyme solution was applied to a Toyopearl HW 55-S (1.6 × 89.0 cm) column equilibrated with 0.1 M phosphate buffer (pH 7) and eluted with the same buffer. The PPO active fractions were pooled and used for enzyme characterization.

Polyacrylamide Gel Electrophoresis (PAGE). Electrophoresis of the purified enzyme was carried out according to the method of Davis (*22*), using 7.5% polyacrylamide gel at pH 9.0.

Molecular Weight Determination. The molecular weight of the purified enzyme was estimated by gel filtration and SDS-PAGE. Gel filtration was conducted on a Toyopearl HW 55-S column (1.6 × 89.0 cm), which was equilibrated and eluted with 0.1 M phosphate buffer (pH 7.0), according to the method of Andrews (*23*). Chymotrypsinogen A (25000), egg albumin (45000), bovine serum albumin (65000), and γ-globulin (125000) were used as marker proteins at the flow rate of 20 mL/h. SDS-PAGE was carried out according to the method described by Weber and Osborn (*24*), using a SDS marker protein kit (Oriental Yeast Co., Tokyo, Japan) as standard.

RESULTS AND DISCUSSION

Enzyme Purification. Enzyme was purified from the homogenates of garland chrysanthemum by ammonium sulfate fractionation, DEAE-Cellulofine, CM-Sephadex C-50, butyl-Toyopearl 650M, and Toyopearl HW 55-S gel filtration. As shown in **Figures 1** and **2**, the enzyme activity showed a sharp single peak on hydrophobic chromatography and also a single peak on final gel filtration. The typical result of stepwise purification of the enzyme is given in **Table 1**. Finally, the enzyme was purified ~32-fold with a recovery rate of 16% as compared with the crude enzyme.

Some Properties of Garland Chrysanthemum PPO. The purified enzyme produced a single band on PAGE and SDS-PAGE (**Figure 3**). These results suggested that garland chrysanthemum PPO was purified to a homogeneous state. As shown in **Figure 4**, the molecular weight of the enzyme was estimated to be about 47000 by gel filtration and 45000 by SDS-PAGE.

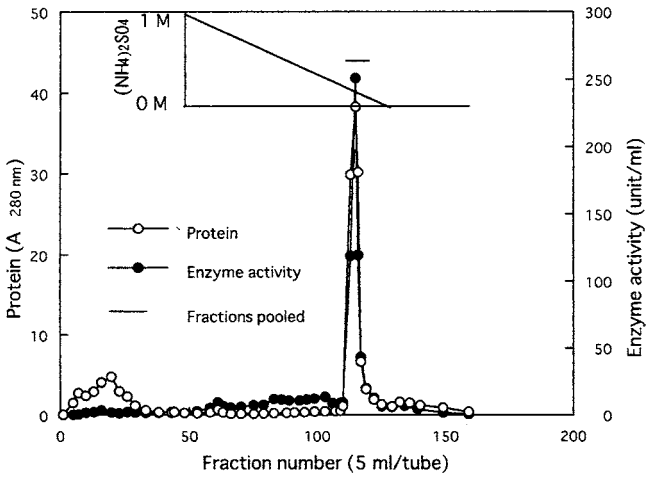

Figure 1. Elution pattern of the enzyme on butyl-Toyoperl 650M: ○, protein; ●, PPO activity; —, fractions pooled.

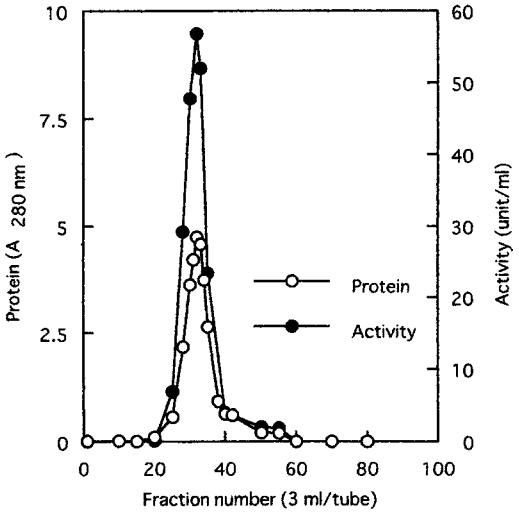

Figure 2. Elution pattern of the enzyme on Toyopearl HW 55S: ○, protein; ●, PPO activity; —, fractions pooled.

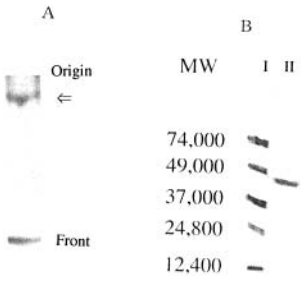

Figure 3. PAGE (A) and SDS-PAGE (B) of the purified enzyme. (A) ⇐, enzyme protein band. (B) I, marker protein; II, purified enzyme; MW, molecular weight.

These results may indicate that the purified PPO is a monomer. The molecular weight of the purified PPO was a little higher than that of banana pulp PPO (*17*), the molecular weight of which was estimated to be about 41000 and 42000 by gel filtration and SDS-PAGE, respectively. The molecular weight of garland chrysanthemum PPO was smaller than that of the PPO of apple (*11*) (MW 65000), Japanese pears (*6*) (MW 56000), and head lettuce (*8*) (MW 56000).

As shown in **Table 2**, the enzyme strongly oxidized chlorogenic acid and (−)-epicatechin. The enzymatic oxidation rates of pyrocatechol and dopamine were ~70% those of chlorogenic acid and (−)-epicatechin. The purified enzyme had no activity

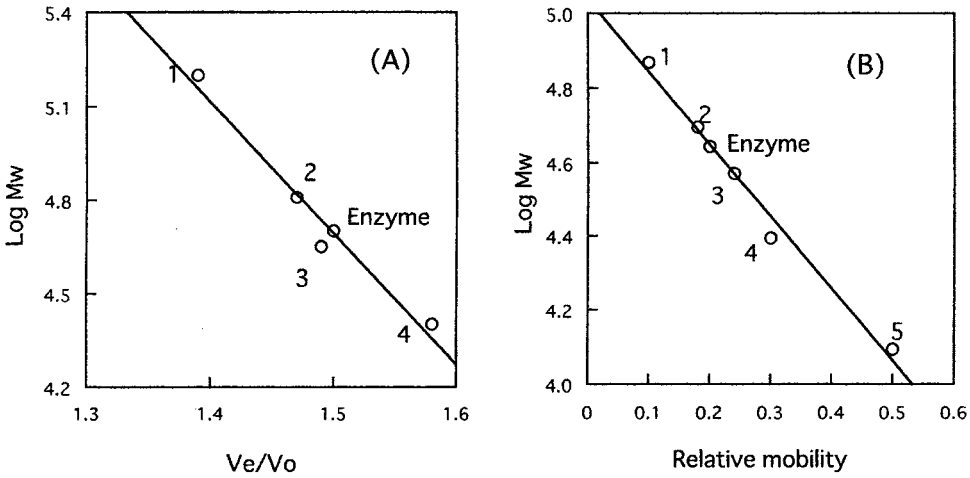

Figure 4. Estimation of molecular weight of the enzyme by gel filtration on Toyopearl HW 55S (A) and SDS-PAGE (B). (A) V_0, void volume of the column; V_e, elution volume of the substance; MW, molecular weight; 1, γ-globulin; 2, bovine serum albumin; 3, egg albumin; 4, chymotrypsinogen A; Enzyme, purified enzyme. (B) MW, molecular weight; cytochrome c: 1, hexamer (74000); 2, tetramer (49600); 3, trimer (37200); 4, dimer (24800); 5, monomer (12400); Enzyme, purified enzyme.

Table 1. Purification of Garland Chrysanthemum PPO

purification step	volume (mL)	total activity (units)	total protein (mg)	specific activity (unit/mg of protein)	purification (fold)	recovery rate (%)
crude enzyme	900.0	16560	5580.0	2.9	1.0	100
DEAE-Cellulofine	140.0	15456	197.7	27.6	9.5	93
CM-Sephadex C-50	127.0	11430	292.0	39.2	13.5	69
butyl-Toyopearl 650M	59.3	4006	70.3	57.0	20.0	24
Toyopearl HW55-S	225.0	2703	29.2	92.6	32.0	16

Table 2. Substrate Specificity of Garland Chrysanthemum PPO

substrate	relative activity (%)
chlorogenic acid	100
(–)-epicatechin	100
pyrocatechol	76
dopamine	74
DL-dopa	72
catechin	70
gallic acid	72
pyrogallol	70
resorcinol	0
phloroglucinol	0

Table 3. Effects of Various Compounds on the Enzyme Activity

compound	relative activity (%) ChO[a] 1 mM[b]	ChO[a] 10 mM[b]	EpO[a] 1 mM[b]	EpO[a] 10 mM[b]
none	100	100	100	100
L-ascorbic acid	0	0	0	0
L-cysteine	4	0	1	0
citric acid	10	3	36	11
acetic acid	15	9	18	9
sodium chloride	25	20	63	41
sodium diethyldithiocarbamate	50	25	–[c]	0
potassium cyanide	30	5	–	0
EDTA	35	21	–	11
$MnCl_2$	3	0	–	5
$BaCl_2$	34	0	-	57
$CuSO_4$	47	11	–	100
$ZnSO_4$	54	16	–	96

[a] ChO and EpO activities were determined at pH 4 and 8, respectively, using McIlvaine buffer. [b] Final concentration of the compound. [c] –, not determined.

on phloroglucinol and resorcinol. These results were mostly similar to those of the reported PPOs, which markedly oxidized such *o*-diphenols as pyrocatechol, chlorogenic acid, and dopamine (*2*, *25*−*27*). The purified garland chrysanthemum PPO strongly oxidized not only chlorogenic acid but also (−)-epicatechin. Similar substrate specificity was found in the PPO of Japanese pears (*6*), lettuce (*8*), and sweet pepper (*28*). The K_m values (Michaelis constant) of the enzyme, measured by using chlorogenic acid (pH 4.0, 30 °C) and (−)-epicatechin as substrate (pH 8.0, 40 °C), were 2.0 and 10.0 mM, respectively. These values were higher than those of lettuce PPO (*8*), which were 0.67 mM for ChO and 0.91 mM for (−)-epicatechin oxidase (EpO), respectively.

Several PPOs show different optimum pH values for different substrates: for example, the pH optima of sweet pepper PPO for chlorogenic acid and pyrocatechol were 4.0 and 7.0, respectively (*28*). The pH optima of lettuce PPO for ChO and EpO (*8*) were at 4.0 and 7.8, respectively. **Figure 5A** shows the effect of pH on ChO and EpO activities of purified garland chrysanthemum PPO. The optimum pH values of the purified PPO were 4.0 and 8.0 for ChO and EpO, respectively. However, purified Japanese pear PPO had a pH optimum of 4.2 for both chlorogenic acid and (−)-epicatechin as substrate (*6*). The acidic pH optima of the PPOs on chlorogenic acid have also been reported for PPOs of potato (*29*), eggplant (*7*), and apple (*11*). Using (−)-epicatechin, pyrocatechol, 4-methylcatechol, and dopamine as substrate, the optimum pH was found at near neutrality for the PPOs of cherry (*3*), avocado (*4*, *5*), banana pulp (*18*), banana peel (*25*), and guava (*27*). The optimum pH of most PPOs for ChO activity was found to be near pH 4 as described above. Therefore, ChOs in these plants are acidic PPOs.

As shown in **Figure 5B**, the activities of ChO and EpO were quite stable in the pH range from pH 5 to 11; ~80% of enzyme activity remained after incubation in various pH solutions from

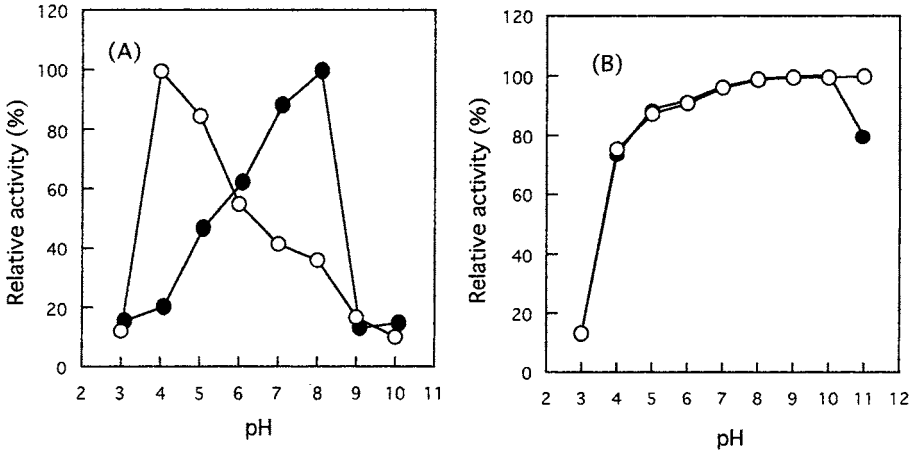

Figure 5. Effect of pH on the activity (A) and stability (B) of the enzyme: ○, ChO activity; ●, EpO activity.

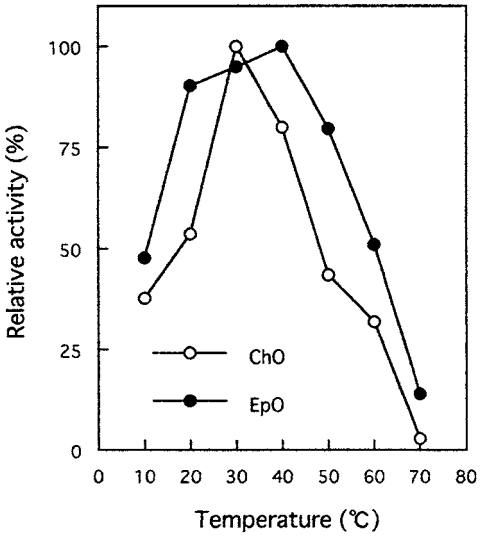

Figure 6. Effect of temperature on the enzyme activity.

3 to 11 for 22 h. The pH stability of the purified enzyme was similar to that of PPOs of banana pulp (*18*), banana peel (*25*), and cabbage (*9*, *10*) but was different from that of PPOs of lettuce (*8*), eggplant (*7*), and apple (*11*).

Figure 6 shows the effect of temperature on the activity of the purified enzyme. The optimum temperatures of enzyme activity were 30 and 40 °C for ChO and EpO, respectively. These temperature optima were a little different from that of the head lettuce PPO (*8*). As shown in **Figure 7**, the activities of ChO and EpO remained unchanged after heat treatment at 50 °C for 10 and 30 min. These results indicated that the enzyme had a relatively high thermal stability. Similar thermal stabilities have been also reported for the PPOs of banana pulp and peel (*18*, *25*), kiwi fruit (*26*), lettuce (*8*), and cabbage (*9*, *10*).

The effects of various compounds on the purified enzyme activity are listed in **Table 3**. The activities of ChO and EpO were markedly inhibited by sodium diethyldithiocarbamate, potassium cyanide, EDTA, and sodium fluoride. Metal ions (Mn^{2+}, Cu^{2+}, and Zn^{2+}) strongly inhibited ChO activity and weakly inihibited EpO at 10 mM. The complete inhibition of ChO and EpO activities was induced by L-ascorbic acid and L-cysteine at 1 and 10 mM, respectively. Similar effects of these compounds were found for PPOs of banana peel and pulp (*9*, *25*, *29*), Japanese pear (*8*), head lettuce (*30*), and cabbage (*9*, *10*). In the case of PPOs in banana peel and pulp (*18*, *25*), their activities were markedly inhibited by citric acid and acetic acid under low buffer capacities (using 0.01 M PB, pH 7) at 10 mM. However, both ChO and EpO of garland chrysanthemum PPO were strongly inhibited by citric acid and acetic acid in high buffer capacity (using McIlvaine buffer, pH 4 for ChO and pH 8 for EpO) at 1 mM. Sodium chloride also markedly inhibited the activities of ChO and EpO.

Considering the results obtained, L-ascorbic acid, L-cysteine, acetic acid, citric acid, and sodium chloride are expected to be used as effective inhibitors of enzymatic browning in garland chrysanthemum.

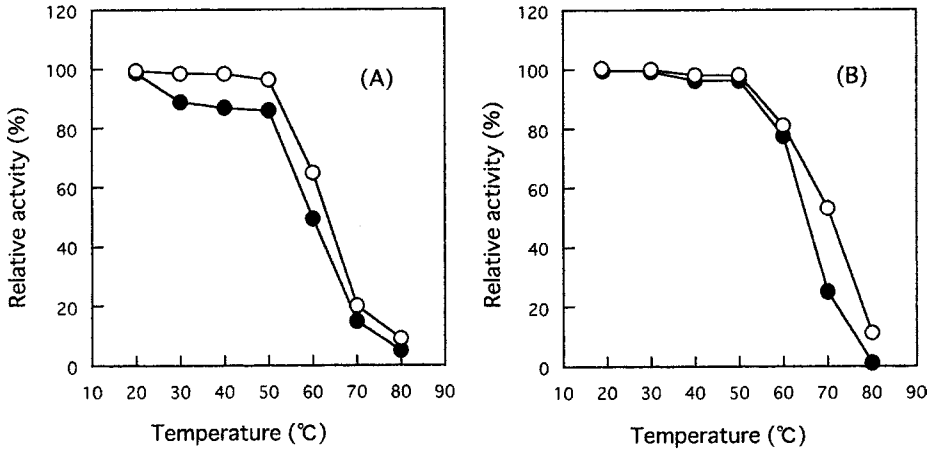

Figure 7. Effect of temperature on the enzyme stability: (A) ChO activity; (B) EpO activity; ○, 10 min; ●, 30 min.

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