The peroxidase activity of cytochrome b_6f complex from spinach chloroplasts

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Abstract

The cytochrome b_6f (Cyt b_6f) complex, which functions as a plastoquinol-plastocyanin oxidoreductase and mediates the linear electron flow between photosystem II (PSII) and photosystem I (PSI) and the cyclic electron flow around PSI, was isolated from spinach (*Spinacia oleracea* L.) chloroplasts using *n*-octyl-β-D-glucopyranoside (β-OG). The preparation was also able to catalyze the peroxidase-like reaction in the presence of hydrogen peroxide (H_2O_2) and guaiacol. The optimal conditions for peroxidase activity of the preparation included: pH 3.6, ionic strength 0.1, and temperature 35ºC. The apparent Michaelis constant (K_m) values for H_2O_2 and guaiacol were 50 mM and 2 mM, respectively. The bimolecular rate constant (k_{obs}) was about 26 M^{-1} s^{-1} and the turnover number (K_{cat}) was about 60 min^{-1} (20 mM guaiacol, 100 mM sodium phosphate, pH 3.6, 25°C, $[H_2O_2]$<100mM). These parameters were similar to those of several other heme-containing proteins, such as myoglobin and Cyt *c*.

Additional key words: cytochrome b_6f complex; guaiacol; heme-containing protein; hydrogen peroxide; peroxidase activity.

Introduction

Cyt b_6f complex is one of the three integrate membrane protein complexes in the photosynthetic electron transport chain. It functions as a plastoquinol-plastocyanin oxidoreductase and mediates the linear electron flow between PSII and PS I and the cyclic electron flow around PSI, thereby contributing to building up a proton gradient across the thylakoid membrane for the generation of ATP (Hope 1993, Vácha *et al*. 2000). The monomeric unit of the ~217 kDa dimeric Cyt b_6f complex contains four main subunits, Cyt *f* (containing one *c*-type heme), Cyt b_6 (two *b*-type hemes and one *c*-type heme), the Rieske iron sulfur protein (one [2Fe-2S]-cluster), subunit IV, and four small subunits, Pet G, Pet L, Pet M, and Pet N. Also, the two prosthetic groups, chlorophyll *a* (Chl *a*) and carotenoid, have been identified in a 1:1:1 ratio with the monomer Cyt b_6f (Kurisu *et al*. 2003, Stroebel *et al*. 2003).

Cyt b_6f complex is also a heme-containing protein complex and shows peroxidase activity, which was reported by Hurt and Hauska (1981) and by Lemaire *et al*. (1986). Many peroxidases are heme-containing enzymes that efficiently catalyse substrate oxidations using H_2O_2. On the other hand, peroxidase activity is inherent to many heme-containing proteins besides peroxidase (Diederix *et al*. 2001). In our previous work, we reported the Cyt b_6f preparation isolated using β-OG showed peroxidase activity and H_2O_2 could induce the oxidation of the Chl *a* bound with the preparation (Chen *et al*. 2006). But the peroxidase activity of Cyt b_6f complex was detected only on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels by 3,3',5,5'-tetramethyl benzidine (TMBZ) staining procedure in previous reports, and the characterization of peroxidase activity was not determined.

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Abbreviations: Chl *a* – chlorophyll *a*; Cyt – cytochrome; H_2O_2 – hydrogen peroxide; HRP – horseradish peroxidase; K_{cat} –turnover number; K_m –Michaelis constant; k_{obs} –bimolecular rate constant; OD – optical density; PS – photosystem; Q_y – red absorption band of porphyrins; TMBZ – 3,3',5,5'-tetramethyl benzidine; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; β-OG – *n*-octyl-β-D-glucopyranoside.
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In the present work, we isolated Cyt b_6f preparation from spinach (*Spinacia oleracea* L.) chloroplasts using β-OG, partially characterized the peroxidase activity of the preparation, determined the influences of pH, ionic strength, and temperature on the activity, and compared the peroxidase activity of the complex with those of other heme-containing proteins.

Materials and methods

Cyt b_6f complex was purified from spinach chloroplasts using β-OG according to the procedure described previously (Yan *et al*. 2001). The Cyt b_6f preparation was resuspended in 50 mM Tricine-NaOH (pH 8.0) containing 30 mM β-OG, and stored at –80°C before use.

Spectroscopic measurements of the Cyt b_6f preparation were carried out on a *UV-Vis* spectrophotometer (*UV-2550, Shimadzu Inc.*, Kyoto, Japan) and the spectral bandwidth was 1.2 nm. Cyt b_6 and Cyt *f* were determined as reported by Mao *et al*. (1998).

SDS-PAGE was performed on 15% polyacrylamide gels in the presence of 6 M urea according to Laemmli (1970), and the proteins in the gels were stained with Coomassie brilliant blue R-250.

TMBZ/H_2O_2 staining of heme-associated peroxidase activity was carried out as described by Thomas *et al*. (1976), and the following procedure was used: 3 parts of 6.3 mM TMBZ solution freshly prepared in methanol were mixed with 7 parts of 0.25 M sodium acetate (pH 5.0). The gels were immersed in this mixture at 25°C in the dark. After 1 h with occasional mixing, H_2O_2 was added to a final concentration of 30 mM. The staining was visible within 3 min.

The peroxidase activity of Cyt b_6f complex using guaiacol as substrate was done according to previous report (Civello *et al*. 1995) with slight modifications. The following reaction mixture was used: 0.1 M sodium acetate buffer (pH 3.6), 30 mM β-OG, 20 mM guaiacol and 1 μM Cyt b_6f sample, in a final volume of 0.5 ml. The assays were typically performed in 0.5 ml quartz cuvettes and the reactions were started by the addition of H_2O_2 after the mixtures were incubated for 2 min at 25°C. The H_2O_2 stock solution was freshly made and its concentration was verified spectrophotometrically (ε_{240} = 39.4 M^{-1} cm^{-1}) (Nelson and Kiesow 1972). The hydrogen donor was guaiacol diluted from a fresh 95% alcohol stock solution (2 M). It was assumed that the coloured product, tetra-guaiacol, was the result of four one-electron oxidations (Baldwin *et al*. 1987). The formation of tetra-guaiacol (ε_{470} = 26.6 mM^{-1} cm^{-1}) (Diederix *et al*. 2001) was followed with spectrophotometry.

Assays at different temperatures were carried out on the spectrophotometer fitted with a thermostat.

Assays at different ionic strengths were carried out by adding suitable amounts of 4 M NaCl to the reaction mixtures.

Assays at different pHs were carried out in different buffer systems: glycin-HCl, pH 2.2 and 3.3; sodium acetate, pH 3.6, 4.0, 4.5 and 5.0; sodium phosphate, pH 6.0 and 7.0; and Tricine-NaOH, pH 8.0.

Data treament: Data about the peroxidase activity shown in the figures were processed using the software (*Origin 7.5 Professional*, *Microcal Inc*., US) and presented as the mean ± SD (n = 5 repetitions).

Results and discussion

Spectroscopic characterization of Cyt b_6f preparation: Fig. 1 shows the absorbance spectra of the purified Cyt b_6f preparation at room temperature. The Chl *a* in the complex shows a Q_y maximum at 669 nm. The shoulder at about 480 nm suggests the presence of a carotenoid. The two main peaks at 421 nm and 431 nm correspond to the Soret bands of Cyt *f* and Cyt b_6, respectively. The two peaks at 523 and 554 nm are attributed to β-band and α-band of Cyt *f*, and the two peaks at 533 and 563 nm are attributed to those of Cyt b_6 (*insert*). The ratio of *f* to *b* heme in the complex was determined from the amplitudes in the Cyt α-band region of the chemical difference spectra, ascorbate minus ferricyanide for Cyt *f* (*insert, dotted line*) and dithionite minus ascorbate for Cyt b_6 (*insert, solid line*), whose ratio was about 1:2, an indication that none of the two *b*-type hemes was lost during purification. These results are also very consistent with the previous reports (Hurt and Hauska 1981, Black *et al*. 1987, Mao *et al*. 1998, Dietrich and Kűhlbrandt 1999).

Peroxidase activity of Cyt b_6f preparation detected on SDS-PAGE gels by TMBZ staining: Cyt b_6f preparation contains four main subunits, Cyt *f*, Cyt b_6, the Rieske iron sulfur protein, and subunit IV, the molecular masses of the subunits from spinach are 33, 23, 20, and 17 kDa, respectively (Fig. 2, *lane 2*). Cyt *f* and Cyt b_6 subunits bind hemes: Cyt *f* contains one *c*-type heme, Cyt b_6 two *b*-type hemes and one *c*-type heme, and both were stained blue with TMBZ/H_2O_2 on SDS-PAGE gel (Fig. 2, *lane 3*). This result is very consistent with the previous report by Hurt and Hauska (1981) and by Lemaire *et al*. (1986).

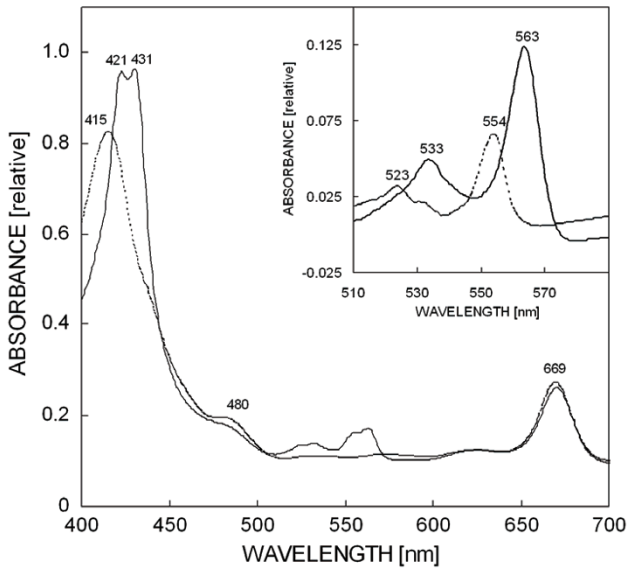

Fig. 1. Absorbance spectra of purified Cyt b_6f preparation reduced by dithionite (*solid line*) and oxidized by ferricyanide (*dotted line*) at room temperature. The peak at 669 nm shows the Q_y maximum of Chl *a*. The shoulder at about 480 nm suggests the presence of a carotenoid. The two main peaks at 421 nm and 431 nm correspond to the Soret bands of Cyt *f* and Cyt b_6, respectively. (*insert*) Redox difference spectrum of the Cyt *b* with maxima at 533 and 563 nm (reduced with dithioite minus reduced with ascorbate, *solid line*) and the Cyt *f* with maxima at 523 and 554 nm (reduced with ascorbate minus oxidized with ferricyanide, *dotted line*).

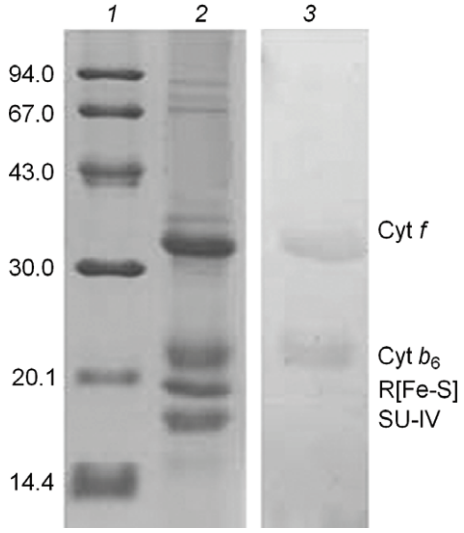

Fig. 2. SDS-PAGE of Cyt b_6f preparation from spinach chloroplasts, using a 15% acrylamide gel. *Lane 1*: markers. *Lane 2*: Cyt b_6f preparation isolated using β-OG, containing four main protein subunits, Cyt *f*, Cyt b_6 , the Rieske iron sulfur protein, and subunit IV. Lane 1 and lane 2 were stained with Coomassie brilliant blue R-250. *Lane 3*: Cyt b_6f preparation stained for peroxidase activity with TMBZ/H_2O_2. The heme-containing proteins, Cyt *f* and Cyt b_6 subunits, exhibited peroxidase activity.

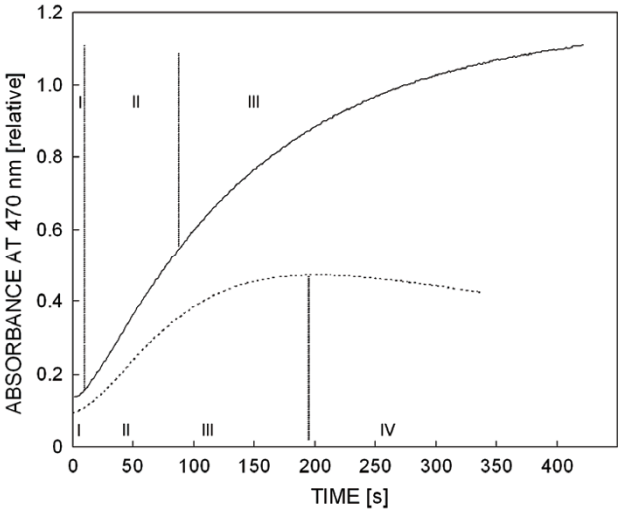

Fig. 3. Typical tetra-guaiacol formation curves given by Cyt b_6f preparation (*straight line*) and Cyt *c* (*dotted line*). The four phases (I–IV) are described in the text. Conditions for Cyt b_6f complex were 40 mM H_2O_2, 20 mM guaiacol, 1 μM Cyt b_6f, 30 mM β-OG, and pH 3.6. Conditions for Cyt *c* were 40 mM H_2O_2, 20 mM guaiacol, 4 μM bovine heart Cyt *c*, and pH 7.6.

Cyt b_6f is heme-containing protein complex and displays peroxidase activity in presence of H_2O_2. It is not surprising because peroxidase activity is showed by many heme proteins besides peroxidase and it has been detected in, *e.g.* hemoglobins, myoglobins, Cyt *c* , and micro-peroxidases (Vazquez-Duhalt 1999, Diederix *et al.* 2001).

The peroxidase activity of Cyt b_6f complex: When Cyt b_6f, guaiacol, and H_2O_2 were mixed together in the reaction buffer, the orange-coloured tetra-guaiacol was formed. When Cyt b_6f or H_2O_2 was left out, no formation of tetra-guaiacol was observed. Mannitol (100 mM) or superoxide dismutase had little effect on the peroxidase activity. Therefore, hydroxyl or superoxide radicals, respectively, are not involved in the reaction, thus excluding Haber-Weiss or Fenton reaction (Radi *et al.* 1991, Diederix *et al.* 2001).

A typical peroxidase activity assay with Cyt b_6f complex and guaiacol gives a product formation curve as depicted in Fig. 3 (*straight line*). An initial activation phase (I of *straight line*) is followed by a steady-state phase (II of *straight line*), represented by the straight part of the curve. Then the curve levels off (III of *straight line*). The rate of the steady-state reaction was determined by taking the maximum of the first derivative of the product formation curve. The length of the steady-state phase (II), as judged from the width of the first derivative maximum, depends strongly on conditions such as [H_2O_2] and pH. Ideally, the length of the steady-state phase was more than 60 s, but frequently it was shorter. The value of peroxidase activity of Cyt b_6f was obtained according to the steady-state phase. The presence of a lag period (phase I) suggests that Cyt b_6f is activated by H_2O_2. The

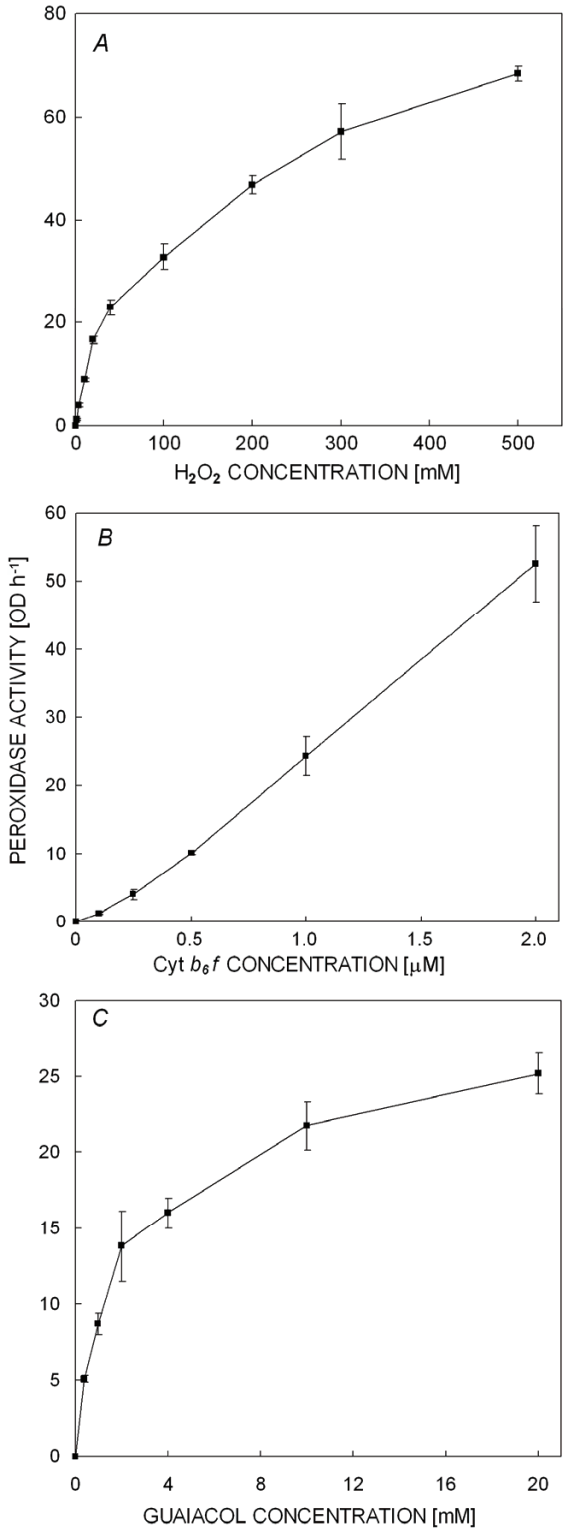

Fig. 4. *A*: Dependence of the peroxidase activity as a function of H_2O_2. Conditons: 1 μM cyt b_6f sample, 20 mM guaiacol. *B*: Dependence of the peroxidase activity as a function of cyt b_6f sample. Conditons: 40 mM H_2O_2, 20 mM guaiacol. *C*: Dependence of the peroxidase activity as a function of guaiacol. Conditons: 40 mM H_2O_2, 1 μM cyt b_6f sample. Mean ± SD; n= 5. OD – optical density.

activation phase was shortened by increased concentrations of H_2O_2. A typical peroxidase activity assay with bovine heart Cyt *c* and guaiacol gives a product formation curve including four phases (Fig. 3, *dotted line*): an initial activation phase (I of *dotted line*), a steady-state phase (II of *dotted line*), a level curve phase (III of *dotted line*), and a decrease phase (IV of *dotted line*). Compared with the activation phase of Cyt *c*, the activation phase of Cyt b_6f is not obvious, which indicates the Cyt b_6f complex is easier to be activated by H_2O_2. The decrease phase is not seen in the tetra-guaiacol formation curve of Cyt b_6f complex under experimental conditions. The phase III and IV features can be ascribed to the inherent instability of the reaction product (tetra-guaiacol), in combination with catalyst inactivation (Diederix *et al*. 2001). This result suggests that the Cyt b_6f complex is more stable than bovine heart Cyt *c* during the tetra-guaiacol formation.

Dependence on hydrogen peroxide, Cyt b_6f and guaiacol: When the concentration of guaiacol is between 0.4 and 2 mM, the peroxidase activity of Cyt b_6f depends linearly on the concentration of H_2O_2 (Fig. 4*A*) and this linear dependence holds up to 100 mM. The rate also depends linearly on the concentration of Cyt b_6f complex (0.1–2 μM) (Fig. 4*B*), and therefore the oxidation rate of guaiacol due to the peroxidase activity of Cyt b_6f follows the bimolecular rate law:

$$\upsilon = k_{obs}\,[\text{Cyt } b_6f]\,[H_2O_2]$$

where υ is the rate of the reaction being catalyzed and k_{obs} is an apparent rate constant.

The value of the bimolecular apparent rate constant k_{obs} was 26 ±1.2 M^{-1} s^{-1} (20 mM guaiacol, 0.1 M sodium phosphate, pH 3.6, 25°C, $[H_2O_2]$<100mM). In this study, the K_m (Michaelis constant) for H_2O_2 was about 50 mM and the saturation was seen up to 100 mM. The magnitude of the K_m corresponding to the binding of H_2O_2 to Cyt b_6f complex reflects a weak affinity because this value can be compared to the values of K_m for horse heart Cyt *c* to be 25–65 mM, which supports absence of specific recognition site for interaction of the peroxide with the Cyt *c* (Radi *et al*. 1991, Hamachi *et al*. 1997, Rosei *et al*. 1998). The guaiacol concentration has a limited effect on the rate (Fig. 4*C*). The peroxidase activity did not increase, however, by increasing concentrations of guaiacol when the concentration of H_2O_2 was 40 mM or relative low. This in fact serves as evidence for a ping-pong mechanism, in which the association with H_2O_2 is rate-limiting. Most peroxidases are relatively nonselective for the reducing substrate indicating that there is no specific recognition site for the substrate at the heme active site. Observation of very similar values of K_m for various heme-containing proteins and the Cyt b_6f complex were shown in Table 1, thus supports nonspecific interaction between the guaiacol and the protein complex. The value of K_{cat} corresponds to the enzymatic

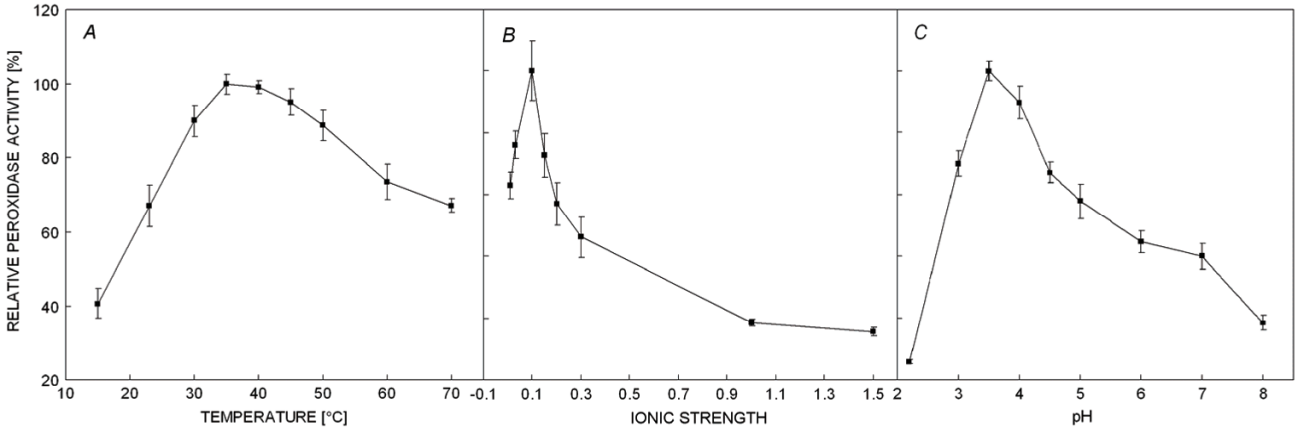

Fig. 5. Effect of temperatures (*A*), ionic strengths (*B*), and pHs (*C*) on the peroxidase activity of Cyt b_6f preparation. Conditions: 20 mM H_2O_2, 20 mM guaiacol, and 1 μM Cyt b_6f sample. Mean ± SD; n=5.

Table 1. Catalytic constants for guaiacol by H_2O_2 catalyzed by various heme-containing proteins.

Enzyme	K_m [M]	K_{cat} [min^{-1}]	K_{cat}/K_m [min^{-1} M^{-1}]	Reference
HRP	5.8×10^{-3}	2.5×10^{4}	4.3×10^{6}	Savenkova *et al.* 1998
Cyt *c* peroxidase	10×10^{-3}	264	2.64×10^{4}	Yonetani 1976
Myoglobin	32×10^{-3}	21.6	1.2×10^{3}	Hayashi *et al*. 1999
Cyt b_{562}	1.2×10^{-3}	0.5	0.4×10^{3}	Mazumdar *et al*. 2003
Cyt b_6f	2×10^{-3}	50	2.5×10^{4}	This work

turnover number. The K_{cat} of Cyt b_6f was about 50 min^{-1} and similar to that of myoglobin (Table 1) (Hayashi *et al*. 1999), but much lower than that of horseradish peroxidase (HRP) (Savenkova *et al*. 1998).

Temperature, ionic strength and pH dependence of the peroxidase activity: Peroxidase activity of Cyt b_6f was measured at different temperatures, pHs, and ionic strengths (Fig. 5*A–C*). A steep increase in enzyme activity is found when the temperature rose from 15 to 35°C, whereas higher temperatures cause slight decrease of the activity (Fig. 5*A*). The activity at 35°C is nearly 1.5-fold higher than the activity at 25°C, and the activity at 70°C is nearly equal to the activity at 25°C, suggesting its high capacity of resistance against heat in peroxidase activity. The different ionic strengths have obvious effects on the activity, and the highest activity is at an ionic strength of μ = 0.1 and higher ionic strength (μ>0.3) inhibits the activity violently (Fig. 5*B*). The Cyt b_6f shows higher peroxidase activities at low pH range than at high pH range. The pH was determined to be 3.6, at which the peroxidase activity of the Cyt b_6f is about 5-fold higher than the activity at pH 8.0 (Fig. 5*C*). So the optimal conditions for the peroxidase activity of Cyt b_6f preparation under experimental conditions included: pH 3.6, ionic strength 0.1, and temperature 35ºC. Many documents reported the experimental conditions for peroxidase activity of peroxidase, Cyt *c* peroxidase, myoglobin, Cyt *c* and Cyt c_{550}, included: pH 6~8, ionic strength 0.1, and temperature 25~30ºC (Orii 1982, Civello *et al.* 1995, Hildebrand *et al.* 1998, Vazquez-Duhalt 1999). The catalytic activities of Cyt *c* (Radi *et al*. 1991) and carboxymethylated Cyt *c* (Prasad *et al*. 2002) were found to be maximum activities in the acidic pH range, similar to the results in this work.

Hence the results presented in this communication demonstrate that the Cyt b_6f preparation isolated from spinach chloroplast using β-OG shows peroxidase-like activity to a certain extent. It has the similar properties of peroxidase activity to those of several other heme-containing proteins. However, the peroxidase activity of the complex is much lower compared with that of HRP. Morevoer, we do not know if the peroxidase activity would be shown *in vivo*.

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