Effect of Temperature on the Activity of Respiratory Enzymes and Oxidative Phosphorylation in Crop Leaves*

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In the previous paper⁸⁾, it was reported that the activity of cytochrome oxidase in the crop roots was inhibited *in vivo* by high temperature above 25~30°C, and the role of phospholipid or phospholipid-protein interaction was anticipated on sustaining the activity of cytochrome oxidase under high temperature. In the present paper, further investigation was done on the relationship between the temperature and the activity of respiratory enzymes in crop leaves.

METHODS AND RESULTS

1. Respiratory enzyme activity under different temperatures

The leaves of rice (*Oryza sativa* L. cv. Tamayodo), Italian ryegrass (*Lolium multiflorum* LAM.) and orchard grass (*Dactylis glomerata* L.) were used as the plant materials. The seeds of Italian ryegrass and orchard grass were purchased from Yukijirushi Shubyō Co. The rice seeds were sown in a conventional seed bed on May 10, and the seedlings were grown for 30~35 days before their enzyme activity was determined. The seedlings of Italian ryegrass and orchard grass, the seeds of which were sown in Wagner pots of 1/5,000 are on September 10, were grown for 50~60 days until their enzyme activity was determined. Preparation of enzymes was carried out under the low temperature of 0~4°C. Preparation of cytochrome oxidase was as follows. Sixty grams of the fresh leaves were washed, cut into piece, and homogenized by a waring blender with 300 ml of 0.1M phosphate buffer (pH 7.4) containing 0.25M sucrose and 0.05M EDTA. The homogenate was screened by cheese cloth and centrifuged at 10³G

for 10 minutes. The resulting precipitate was discarded and the supernatant was further centrifuged at 10⁴G for 20 minutes. The precipitate obtained was suspended on the 50 ml of 0.1M phosphate buffer (pH 7.4) containing 0.25M sucrose and centrifuged again at 10⁴G for 20 minutes. The resulting precipitate was suspended on 4.0 ml of the above-mentioned phosphate buffer and offered to the determination of the enzyme activity.

Preparation of ascorbic acid oxidase was made by the following procedure. Five grams of fresh leaves were homogenized with 100 ml of 0.2M phosphate buffer (pH 5.6). The homogenate was screened by cheese cloth and centrifuged at 10⁴G for 20 minutes. The supernatant obtained was offered to the determination of the enzyme activity.

Peroxidase was prepared from 1.0 g of the

Fig. 1. Cytochrome oxidase activity at the different temperatures.

Notes: —●— Italian ryegrass
—○— Orchard grass
—△— Rice

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fresh leaves homogenized in a mortar with 9 ml of 0.1 M phosphate buffer (pH 7.0) and a small amount of quartz sand. The homogenate was centrifuged at 3,000 rpm for 3 minutes and the resulting supernatant was offered to the determination of peroxidase activity.

The determination of the enzyme activity was made by polarographic method^{1,3,5}, except for the determination of peroxidase activity which

Fig. 2. Activity of ascorbic acid oxidase and peroxidase from rice leaves at the different temperatures.

Notes: —○— Ascorbic acid oxidase
—●— Peroxidase

Table 1. Effect of high temperature on the activity of cytochrome oxidase *in vitro*.

	Temperature treatment	Time of temperature treatment		
		0 hr	1 hr	2 hr
Rice	Cont.	24.0	23.0	24.0
	30°C	—	22.0	23.6
	35°C	—	17.4	7.1
	40°C	—	8.8	2.9
Italian ryegrass	Cont.	33.0	35.1	34.2
	30°C	—	27.6	21.4
	35°C	—	21.9	8.5
Orchard	Cont.	56.8	57.2	57.0
	25°C	—	56.3	57.5
	30°C	—	48.6	42.8
	35°C	—	40.6	15.5

Note: The unit of enzyme activity is oxygen uptake in mμM/fresh wt./min

was determined by guajacol method⁶. Followings are the reaction mixtures:

Cytochrome oxidase

0.1 M phosphate buffer (pH 7.4) containing
0.4 M sucrose 1.5 ml
0.1 M hydroquinone aq. sol. 0.2 ml
1.0% cytochrome c(Sigma Chem. Co.)
aq. sol..... 0.1 ml
enzyme preparation 0.2-0.4 ml

Ascorbic acid oxidase

0.2 M phosphate buffer (pH 5.6) ... 1.6 ml
0.1 M ascorbic acid aq. sol..... 0.2 ml
enzyme preparation 0.2 ml

Peroxidase

0.2 M acetate buffer (pH 5.0) 10.0 ml
0.1% guajacol aq. sol. 1.0 ml
0.08% hydrogen peroxide aq. sol. 1.0 ml
enzyme preparation 0.1 ml
water 17.9 ml

The activity of cytochrome oxidase at the different temperatures is shown in fig. 1. The activity of the enzymes from Italian ryegrass and orchard grass linearly increased according to the rise of temperature, while in rice plant the activity of the enzyme increased slowly below 20°C and steeply above 20°C according to the rise of temperature. This behaviour of the rice cytochrome oxidase was also different from those of the rice ascorbic acid oxidase and peroxidase (fig. 2). The activity of the ascorbic acid oxidase and peroxidase from rice leaves linearly increased according to the rise of temperature.

2. Effect of high temperature *in vitro* on the activity of cytochrome

Cytochrome oxidase from the leaves of rice plant, Italian ryegrass and orchard grass was exposed *in vitro* to high temperature above 30°C. Immediately after the enzyme preparation, the enzyme was exposed to high temperature in a water bath for one or two hours and then its activity was determined. During the high temperature treatment, control was stored at 5~7°C in a refrigerator.

Results are shown in table 1. The activity of rice cytochrome oxidase was hardly influenced by the treatment of 30°C, although the treatment of 35°C or 40°C markedly decreased the enzyme activity. In Italian ryegrass and orchard grass, however, the treatment at even 30°C considerably decreased the enzyme activity.

3. Effect of surfactant on the activity of cytochrome oxidase at the different temperatures

Cytochrome oxidase from the leaves of rice plant, Italian ryegrass and orchard grass, was prepared as mentioned above and treated with polyoxyethyleneglycol alkylphenyl ether (Triton X 114 of Dow Chem. Co. or Emulgen 810 of Kao-Atlas Co.) after Jacobs and Andrews⁴⁾. According to them, this surfactant "solubilizes" the crude preparation of cytochrome oxidase which is a phospholipid-protein complex.

Results are shown in fig. 3. According to the rise of temperature, the activity of cytochrome oxidase from every species of the crops linearly increased up to 30°C and decreased above 30°C. The difference between rice and the other

Fig. 3. Effect of surfactant on the activity of cytochrome oxidase at the different temperatures.

Notes: —●— Italian ryegrass
—○— Orchard grass
---△--- Rice

species, which was clearly seen in fig. 1, almost disappeared in fig. 3.

Table 2 shows the effect of surfactant on the thermostability of the rice cytochrome oxidase. The activity of the enzyme pretreated with the surfactant was remarkably decreased by the high temperature treatment, whereas the activity of the enzyme untreated with the surfactant was hardly influenced by the high temperature treatment.

4. Effect of temperature on the mitochondrial oxidative phosphorylation in rice and maize

Mitochondria were prepared after Verleur and Uritani⁹⁾ from etiolated rice (*Oryza sativa* L. cv. Nōrin No. 29) and maize (*Zea mays* L. cv. Chōkō No. 207) seedlings which were grown under 25°C for 5~7 days. Preparation of mitochondria was carried out under 0~4°C. The fresh seedlings of 120 mg were homogenized in a mortar with 200 ml of extracting medium (pH 7.2) containing 0.6 M mannitol, 0.01 M KCl, 0.01 M EDTA, 0.004 M cysteine, 0.05 M tris-HCl, 3.0% polyvinyl pyrrolidone and 0.03 M K-citrate. During the homogenization, hydrogen ion concentration was monitored by pH meter and corrected by dropping 1.0 M tris (hydroxymethyl) aminomethane aqueous solution. The homogenate was screened by cheese cloth, and centrifuged at 10³G for 15 minutes. The resulting supernatant was centrifuged at 1.5×10⁴G for 15 minutes, and the precipitate obtained was suspended on the same solution as the extracting medium, except for the absence of EDTA. The suspension was

Table 2. Effect of surfactant on the thermostability of cytochrome oxidase from rice leaves.

Pretreatment by surfactant	High temp. treatment	Cytochrome oxidase activity
Cont.	Cont.	30.2 (100)
	Treat.	30.0 (99.4)
Treat.	Cont.	123.6 (100)
	Treat.	3.2 (2.6)

Notes: The enzyme was exposed to 40°C for 30 min. The unit of enzyme activity is oxygen uptake in mμM/fresh wt./min

Fig. 4. Mitochondrial oxidative phosphorylation under the different temperatures.

Notes: —●— Oxidative phosphorylation
 —■— State 3 respiration (O_2 uptake in the presence of ADP)
 —□— State 4 respiration (O_2 uptake in the absence of ADP)

centrifuged again at $1.5 \times 10^4 G$ for 15 minutes, and the resulting precipitate was suspended on 8 ml of the medium (pH 7.2) containing 0.4 M mannitol, 0.01 M KCl, 0.05 M tris-HCl and 0.1% bovine serum albumine.

Oxidative phosphorylation was determined by polarographic method^{1,11)}. Rate of oxygen consumption of the reaction mixture, the contents of which were 0.1~0.2 ml of the mitochondrial preparation and 2 ml of reaction medium (pH 7.2, containing 0.4 M mannitol, 0.01 M KCl, 0.05 M tris-HCl and 0.1% bovine serum albumine) containing K-malate at the concentration of 30 mM, was determined in the presence and absence of 50 μ moles of 2Na-ADP.

Results are shown in fig. 4. The efficiency of oxidative phosphorylation which was expressed by ADP/O was remarkably declined above 30°C. Below 25°C, it was also appreciably decreased. According to the rise of temperature, the rate of oxygen consumption increased slowly below 20°C and steeply above 20°C. This change in the rate of oxygen consumption was somewhat similar to that of the rice cytochrome oxidase in fig. 1.

DISCUSSION

It is well known that cytochrome oxidase *in vivo* is a phospholipid-protein complex^{4,10,12)}. In this paper, it was observed that the difference in the temperature response between the rice cytochrome oxidase and that from Italian ryegrass or orchard grass was almost disappeared by the treatment of surfactant which "solubilized" crude cytochrome oxidase. In addition, the rice cytochrome oxidase also different in temperature response from the rice ascorbic acid oxidase and peroxidase which were not a lipoprotein. Besides, the rice cytochrome oxidase was deprived by the surfactant of its thermostability. In consequence, it is inferred that the difference in temperature response between the rice cytochrome oxidase and that from Italian ryegrass or orchard grass is attributable to the difference in the properties of lipid between rice and the others.

No investigation accounting for the decline of oxidative phosphorylation under high or low temperature was made in this paper. However, Lyons et al⁷⁾ observed that swelling and

contraction of mitochondria from chilling sensitive plants were inhibited by chilling temperature. According to the review by Green and Fleisher²⁾, phospholipid in mitochondria plays an important role in the structure and function of mitochondria or mitochondrial enzyme systems. Accordingly, it will be profitable for further investigation on the temperature response of mitochondrial oxidative phosphorylation to presume that the properties of mitochondrial lipid will be concerned.

SUMMARY

The activity of cytochrome oxidase from the leaves of Italian ryegrass and orchard grass linearly increased according to the rise of temperature from 10 to 40°C. The same was true for the ascorbic acid oxidase and peroxidase from rice leaves. However, the activity of cytochrome oxidase from rice leaves increased slowly below 20°C and steeply above 20°C.

After the treatment with the surfactant which "solubilized" the cytochrome oxidase, the above-mentioned difference in temperature response between the rice cytochrome oxidase and that from Italian ryegrass or orchard grass almost disappeared. In addition, the treatment with the surfactant deprived the cytochrome oxidase of its thermostability.

Mitochondrial oxidative phosphorylation from etiolated rice and maize seedlings declined remarkably above 30°C and appreciably below 25°C. The temperature response of the mitochondrial oxygen consumption was somewhat similar to that of the rice cytochrome oxidase.

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〔和 文 摘 要〕

作物葉の呼吸酵素および酸化的リン酸化反応に対する温度の影響

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水稲, イタリアンライグラス, オーチャードグラスの葉のチトクロームオキシダーゼ活性を, 10~40°C の温度で測定した. イタリアンライグラスおよびオーチャードグラスでは, 温度の上昇に従って酵素活性が直線的に上昇したが, 水稲では, 20°C 以下では温度上昇による活性増加率が小さかった. 水稲のアスコルビン酸オキシダーゼおよびパーオキシダーゼは, 温度上昇に従って酵素活性が直線的に上昇した.

チトクロームオキシダーゼを可溶化する界面活性剤で処理すると, 上述の作物による差異はみとめられなくなった. また, 界面活性剤処理によつて, チトクロームオキシダーゼの高温下での安定性が失われた. 以上のことから, 水稲とイタリアンライグラスまたはオーチャードグラスの間におけるチトクロームオキシダーゼの温度反応の差異は, これら作物間における脂質の性質の差異の影響を受けているものと推測した.

水稲およびトウモロコシの黄化幼植物から調製したミトコンドリアの酸化的リン酸化反応を測定した結果, 30°C 以上の温度では ADP/O の比が著しく低下し, 25°C 以下でも低下する傾向があることがみとめられた.