Studies on the Succinoxidase System of *Begonia* Leaves

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Attention has been paid to the problem as to whether oxidative enzymes involved in the TCA cycle are present in chloroplasts as well as in mitochondria of green cells, or they are localized solely in the mitochondria. No definite answer to this question, however, seems to have been given.

McCledon¹⁾ reported that in tobacco leaves cytochrome oxidase was mainly associated with the mitochondria, while catalase and phenolase were found to be present in all fractions obtained from the leaf homogenate.

Brown and Good²⁾ stated that spinach chloroplasts were unable to reduce directly molecular oxygen to form water, except the case in the presence of phenolase, catechol and cytochrome c. James and Das³⁾ who studied the diotone-treated pure chloroplasts of spinach, stated that oxidative enzymes were not present in the chloroplasts.

Brummond and Burris⁴⁾ found that chloroplast fragments possess the activities of oxidative enzymes involved in the TCA cycle, cytochrome oxidase and cytochrome photo-oxidase, and that they were capable of carrying out the Hill reaction. Vernon and Kamen⁵⁾, Ohmura⁶⁾, and Nieman and Vennesland⁷⁾ indicated the possibility of presence of the enzymes of the TCA cycle as well as those involved in the oxidative and photosynthetic phosphorylations in the chloroplasts of spinach. Sissakian^{8,9)} reported the presence of cytochrome oxidase, succinic, malic, and glutamic dehydrogenases in the chloroplasts of sugar beet. Zellitch and Barber¹⁰⁾ found that the chloroplasts prepared in a homogenizing medium which contained 0.45 M sucrose, mannitol, and versen, possessed the activities of diphosphopyridine nucleotide-linked malic dehydrogenase, glycolic acid oxidase, and glyoxylic acid reductase, and stated that the conflicting views on the presence of oxidative enzymes in chloroplasts were due to different methods of preparing the chloroplast fraction; for example, glycerol used as a homogenizing medium caused the inactivation of oxidases.

In this work attempts are made to elucidate some properties of the enzymes involved in the TCA cycle, especially of the succinoxidase system in the particulate fractions of *Begonia* leaves.

Material and Methods

Material: Leaves of *Begonia semperflorens* grown in green house were used.

Preparation of particulate and soluble fractions: Fresh leaves were washed, chopped into pieces, and homogenized in a cold mortar. The homogenizing medium was 0.01M phosphate buffer (pH 7.2) supplemented with 0.005 M cysteine or 0.5M sucrose. The homogenate was filtered through two layers of cheesecloth and centrifuged at $500\times g$ for 3 minutes to remove the cell debris. The supernatant was successively centrifuged for 20 minutes each at 1,500, 8,000, 10,000 to 12,000, and $24,000\times g$. Each sediment was washed twice with the medium, and resuspended in the same medium. Microscopic examination revealed that Sediment-1 (obtained at $1,500\times g$) consisted mainly of whole chloroplasts, and Sediment-2 ($8,000\times g$ -sediment)

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comprised chloroplast fragments. The pale green Sediment-3 (obtained at 10,000 to 12,000 $\times g$) consisted mainly of Janus Green B-staining particles (mitochondria). Sediment-4 (at 24,000 $\times g$) seemed to be composed of the smaller fragments of chloroplasts. To the supernatant obtained after the centrifugation at 24,000 $\times g$, ammonium sulfate was added to 5 per cent-saturation. After removing the precipitates produced, ammonium sulfate was further added to make the solution to 10, 20, 50, and 100 per cent-saturation. From the precipitates thus produced the oxidative enzymes described in the text were extracted with deionized water or 0.01M phosphate buffer.

Treatment of particulate fraction with deoxycholate: The particulate fractions were treated with 0.3 per cent deoxycholate at 30° for 2 hours, or in refrigerator overnight, and centrifuged at 10,000 $\times g$ for 20 minutes. The residue was washed twice with deionized water and suspended in the same medium.

Treatment of particulate fraction with digitonin: The particulate fractions were extracted three times successively with 5 ml of 1 per cent digitonin at 0°C, (first extraction 20 minutes; the second 40 minutes; the third 2.5 hours). After each extraction the sample was subjected to centrifugation at 15,000 $\times g$ for 20 minutes. These extracts were designed as Extract 1, 2, and 3, respectively⁽¹⁾. The residue was washed twice with deionized water.

Treatment of particulate fraction with lipase: Lipase (Tokyo Kasei Kogyo Co.) was added to Sediments 1 and 2 suspended in 0.01M phosphate buffer (pH 7.2) (1 mg of lipase per 100 mg of the particulate protein). The mixture was kept at 30° for 20 minutes or at room temperature (about 20°) for 2 hours. The suspension was subsequently centrifuged at 10,000 $\times g$ for 20 minutes. In some experiments, the treatment was repeated three times. The supernatants thus obtained were designated as Extract 1, 2, and 3. The residue was washed twice with deionized water and suspended in the water.

Assay of activity of oxidative enzymes: The activities of oxidative enzymes were determined by the standard Warburg manometric method. The reaction medium contained enzyme preparation, 0.02M phosphate buffer, and 0.02 M substrate (neutralized) in a total volume of 1.0 or 2.0 ml. In some experiments, cytochrome c (Sigma Chemical Co., Type II, to the amount of 1 mg) or each of the inhibitors listed in Table 7 was added to the basal medium. The reaction was allowed to proceed at 30° for 10 or 20 minutes after the temperature equilibration requiring 5 to 19 minutes. When the reaction was prolonged, the reaction rate was found to be considerably reduced on account of the low stability of the enzymes, especially those in the particulate fraction. The activity was expressed in μ l of oxygen consumed per hour per mg dry weight of particulate fraction or mg of protein of the solubilized enzyme preparations.

Determination of protein: The determination was made by the method of Neilson⁽²⁾.

Results

Activities of succinoxidase, phenolase, and oxalic acid oxidase of fractions separated in two different media and effect of ageing of particulate fractions: In this experiment, phenolase and oxalate oxidase were assayed as the examples of the enzymes which were largely present in chloroplasts and solubilized, the former easily⁽³⁾ and the latter with difficulty. As seen in Table 1, succinoxidase activity was stronger in Sediment-4 than in the other fractions when fractionated in 0.01M phosphate buffer or in 0.5M sucrose. The strongest phenolase activity, however, was found in

Sediment-1 obtained from 0.5 M sucrose-homogenate and in the supernatant obtained at $24,000\times g$ from phosphate buffer-homogenate. The fractions precipitated with ammonium sulfate at 100 per cent saturation from the supernatant, which were obtained in both 0.5 M sucrose and phosphate buffer, oxidized succinate and pyrogallol with high value. The activity of Sediments-1, 2, and 3 was stimulated by ageing the fractions.

Activities of enzymes involved in TCA cycle in chloroplast: Sediments 1 and 2 were able to oxidize succinate, fumarate, malate, and α -ketoglutarate (Tale 2). These activities were stimulated on ageing the particulate suspensions in a refrigerator. The oxidation of malate and α -ketoglutarate was stimulated by the addition of diphospho-

Table 1. Oxidation of succinate, oxalate, and pyrogallol by several fractions separated in two different media and effect of ageing on the activity. Reaction pH, 5.0 (succinate and oxalate) and 6.8 (pyrogallol). $(\text{NH}_4)_2\text{SO}_4$ -precipitate was obtained from supernatant ($24,000\times g$) at 100% saturation and extracted with deionized water.

Homogenizing media	Fraction		QO ₂		
			Succinate	Oxalate	Pyrogallol
0.5 M sucrose	Sediment-1 ($1,500\times g$),	Fresh	1	13	222
		Aged	5		
	Sediment-2 ($8,000\times g$),	Fresh	1	9	95
		Fresh	1	5	1
	Sediment-4 ($24,000\times g$),	Fresh	18	5	3
		Fresh	46	<1	17
	(NH ₄) ₂ SO ₄ -precipitate,	Fresh	147	<1	5
0.01 M phosphate buffer (pH 7.2)	Sediment 1 ($1,500\times g$),	Fresh	10	81	25
		Aged*	13	91	
	Sediment 2 ($8,000\times g$),	Fresh	56	28	15
		Aged*	>56	23	
	Sediment 3 ($12,000\times g$),	Fresh	4	48	21
		Aged*	26	100	
	Sediment 4 ($24,000\times g$),	Fresh	105	46	70
		Aged*	56	28	15
	Supernatant ($24,000\times g$),	Fresh	100	<1	285
		(NH ₄) ₂ SO ₄ -precipitate, Fresh	316	1	137

* Particulate fractions were aged at 0° overnight.

Table 2. Oxidation of succinate, fumarate, malate, and α -ketoglutarate by particulate fractions. Reaction pH, 5.5. Each fraction was obtained from 0.5 M sucrose-homogenate.

Fraction	DPN	QO ₂			
		Succinate	Fumarate	Malate	α -Keto-glutarate
Sediment-1 ($1,500\times g$) Fresh Aged (0°, overnight)	—	1	5	9	—
	—	5	71	9	11
	+			36	19
Sediment-2 ($8,000\times g$) Aged (0°, overnight)	—	11	18	11	—
	+			38	8

pyridine nucleotide (prepared from Baker's yeast).

Extraction of succinoxidase from particulate fractions: Sediment 1 was kept at 0° overnight in three different media: 0.25M sucrose, 0.25M sucrose plus 0.3 per cent deoxycholate, and 0.25M sucrose plus 1 per cent digitonin (final concentration). The treatment with deoxycholate or digitonin resulted in partial extraction of succinoxidase and strong inhibition of the activity of pyrogallol oxidizing enzyme (Table 3). It must be mentioned that the sum of the total succinoxidase activity of the extract and residue was much higher than the total activity of the original non-treated fraction, indicating that remarkable activation of the enzyme in the fraction was brought about by these treatments.

Table 3. Effects of deoxycholate and digitonin on extraction of succinoxidase from chloroplast fraction. Reaction pH, 7.2 (succinate) and 6.8 (pyrogallol). Enzyme preparations were obtained from Sediment-1 of 0.5 M sucrose-homogenate.

Preparation	Total protein (mg)	Activity-I		Activity-II	
		(μl, O ₂ uptaken/hr./mg of protein)		(Activity-I × total mg of protein)	
		Succinate	Pyrogallol	Succinate	Pyrogallol
Non-treated sediment	64	1	38	64	2439
0.25 M sucrose -extract	0.2	59	136	12	27
-residue	63.8	23		1518	
0.3% deoxycholate-extract	4.7	10	22	47	103
-residue	50.0	36	±	1800	±
1% digitonin -extract	0.8	51	1	42	1
-residue	58.5	25	+	1462	±

After the treatment of Sediment-1 and 2 with lipase, the assay for succinoxidase and oxalic acid oxidase were made on the separated clear extracts and residues (Table 4). In this case, oxalic oxidase was assayed as an example of the enzyme present in chloroplasts and of the enzyme solubilized with difficulty (unpublished). Succinoxidase was found, for the most part, in the lipase extract with high activity. It is to be noted that the total activity of the extract and residue was larger than the original activity of non-treated preparation. Sediment-2 was subjected to sonication (Kubota Sonic Vibrator, 9 kc, 5 minutes), which brought about partial extraction of succinoxidase.

Fractionation of succinoxidase and phenolase with ammonium sulfate: Attempts were made to fractionate succinoxidase, phenolase, and oxalic oxidase in the lipase-extracts of chloroplast fractions (Sediments-1 and 2) and in the supernatant at 24,000×g-centrifugation, using ammonium sulfate. It was found that succinoxidase was largely precipitated by the addition of ammonium sulfate at 100 per cent saturation. Oxalic oxidase was localized in the precipitates at 10 per cent saturation (Table 5). From the lipase extracts of chloroplast fractions, at least, two different precipitates were separated at 100 per cent saturation of ammonium sulfate by differential centrifugation; the first precipitate was sedimented by centrifugation at 10,000×g,

while the second precipitate was floating on the surface of the solution at $10,000\times g$ and could be sedimented at $13,000\times g$. Succinoxidase was specifically localized in the latter fraction and catecholase and oxalic oxidase were present predominantly in the former fraction.

Optimum pH for oxidation of succinate: The optimum pH for oxidation of succinate by Sediment-1 was 5.0. The value was the same in the oxidation of the other organic acids in the Krebs cycle, and was different from that observed with the supernatant at $24,000\times g$ which was about 4.5.

Optimum concentration of succinate: The optimum concentration for oxidation by Sediment-1 was about 0.025 M at pH 7.2.

RQ value of succinate oxidation: The RQ values observed with the preparations

Table 4. Effects of treatment with lipase on extraction of succinoxidase from chloroplast fraction. Reaction pH, 5.0. Reaction time, 10 minutes. Fractions were obtained from 0.5 M sucrose-homogenate.

Fraction	Total dry weight* (mg)	Activity-I		Activity-II	
		(μl, O ₂ uptaken/hr./mg of protein)		(Activity-I × total mg of protein)	
		Succinate	Oxalate	Succinate	Oxalate
Sediment-1 Non-treated	20.0	5	38	100	460
Lipase extract-1	3.3	74	76	244	251
extract-2	3.8	153	—	581	—
extract-3	0.9	317	124	285	112
residue	12.0	105	—	126	—
Sediment-2 Non-treated	20.0	11	15	220	300
Lipase extract-1	1.7	122	125	206	213
extract-2	2.2	22	—	48	—
residue	12.0	33	72	298	864

* Total protein was measured in the experiments with lipase-extract.

Table 5. Fractionation of succinoxidase with ammonium sulfate.

Fraction	QO ₂		
	Succinate	Oxalate	Catechol
Supernatant (24,000 × g), 5% sat. (NH ₄) ₂ SO ₄ -ppt*	32	<1	81
10% sat. "	37	19	39
20% sat. "	392	<1	—
50% sat. "	288	<1	137
100% sat. "	1090	<1	135
Sediment-1, Lipase extract-1, (NH ₄)SO ₄ -ppt-1**	<1	176	940
-2**	36	7	<1
Lipase extract-2, (NH ₄)SO ₄ -ppt-1**	<1	368	170
-2**	380	<1	<1
Sediment-2, Lipase extract-2, (NH ₄)SO ₄ -ppt-1**	16	298	525
-2**	190	25	32

*, ** (NH₄)₂SO₄-precipitates were extracted with deionized water. ** (NH₄)₂SO₄-precipitate-1 was sediment at $10,000\times g$, and precipitate-2 was sediment at $13,000\times g$. The precipitates were obtained at 100% saturation of (NH₄)₂SO₄.

obtained from the $24,000\times g$ -supernatant at 50 and 100 per cent saturation of ammonium sulfate were 3.0 and 1.5, respectively.

Effect of cytochrome c on oxidation of succinate: The activities of succinoxidase in Sediments-1 and 3 and Supernatant (cytoplasmic) fraction were stimulated by the addition of cytochrome c (Table 6). The extract of the precipitate obtained from the cytoplasmic fluid at 100 per cent-saturation of ammonium sulfate indicated the

Table 6. Effect of cytochrome c on oxidation of succinate by several fractions.

Fraction	Reaction system		QO ₂ pH	
	Succinate	Cytochrome c	5.5	7.2
Sediment-1	—	—	11	
	—	+	21	
	+	—	21	
	+	+	32	
Sediment-3	—	+	<10	<1
	+	+	129	89
100% sat. (NH ₄) ₂ SO ₄ -ppt* 0.01 M phosphate buffer-extract -residue	+	—	15	8
	+	+	20	14
	+	—	16	12
	+	+	<1	5

* The precipitate was obtained from the supernatant at $24,000\times g$ -centrifugation.

Table 7. Effects of several inhibitors on activity of succinoxidase in various fractions.

Fraction	Reaction pH	Inhibition (%)** by						
		KCN			NaN ₃	PCMB	Cl-pro.	Malon.
		1×10^{-3} (M)	2×10^{-3} (M)	1×10^{-2} (M)	2×10^{-3} (M)	1×10^{-5} (M)	5×10^{-4} (M)	5×10^{-2} (M)
Sediment-1	7.2	100	100	100				
Sediment-2	7.2	98	100	100	67			
Sediment-3	7.2	100	100	100	30			
Sediment-4	7.2	76		78				
Supernatant ($24,000\times g$)	7.2	26	30	56				
100% sat. (NH ₄) ₂ SO ₄ -ppt (from Supernatant)	7.2	21	18	35	37			
	5.0	0	0	10			37	59
Sonic-extract (from Sediment-2)	7.2	100	100					
Extract of Sediment-2*	6.8					41		
	5.0					20		
Residue of Sediment-2*	6.8					53		
	5.0					9		

* Extract and residue were obtained by ageing of Sediment-2 in 0.01M phosphate buffer at 0° for 24 hours.

** PCMB, *p*-chloromercuribenzoate; Cl-pro., chlorpromazine; Malon., malonate.

stimulation, while the residue did not.

Effect of enzyme inhibitors: The activity of succinoxidase in the particulate fractions was strongly inhibited by cyanide and azide, while the enzyme activity in the supernatant at $24,000\times g$ -centrifugation was affected to much less extent at the same concentration of the inhibitors (Table 7). The degree of inhibition was stronger at pH 7.2 or 6.8 than at 5.0. Chlorpromazine, malonate, or *p*-chloromercuribenzoate produced the partial inhibition.

Discussion

All the fractions of leaf homogenate tested indicated the activity of succinoxidase. Of the particulate fractions, sediment-4, the smaller fragments of chloroplasts, perhaps mixed with mitochondria, oxidized succinate indicating the highest activity. The activity of the whole chloroplasts was strikingly increased by ageing. It was demonstrated that succinoxidase system was hardly solubilized by 0.01 M phosphate buffer or 0.5 M sucrose from the particulate fractions while phenolase was easily resolved with 0.01 M phosphate buffer but not with 0.5 M sucrose. The striking solubilization and activation of succinoxidase of chloroplast fractions by treatment with lipase suggested that there exists certain mechanism of masking of the enzyme activity in the particles*. It appeared that succinoxidase system together with phenolase was present in the chloroplast fraction, and the segmentation to smaller fragments or ageing of chloroplasts brought about the activated state of the enzyme system.

The total activity of succinoxidase in the fractions separated from the cytoplasmic fluid with ammonium sulfate was very low. There are two possibilities; either that this small amount of the enzyme was originally contained in the soluble form, or that it was extracted from the particulate fractions during the homogenation procedures.

The experiments on oxidation of the Krebs cycle intermediates by the particulate fractions demonstrate the presence of enzymes involved in this cycle in the particulate fractions.

The acidic optimum pH for oxidation of intermediates of the Krebs cycle by the various fractions may be unique property of *Begonia* leaves. The property may perhaps be related to the strongly acidic pH in cells due to the presence of large amounts of free organic acids. The activity of succinoxidase of various fractions was stimulated by the addition of cytochrome c, and was inhibited by cyanide and azide just as the activity of cytochrome c oxidase is. It is probable that the oxidation of succinate by various fractions of leaf homogenate was mediated by cytochrome c and cytochrome c oxidase.

Summary

Several properties of succinoxidase and other oxidizing enzymes in the chloroplasts and various other fractions of leaf homogenate of *Begonia semperflorens* were investigated.

1. Succinate, fumarate, malate, and α -ketoglutarate were oxidized by the various fractions of leaf homogenate. The activities in the particulate fractions were stimulated by ageing of these fractions.

* The same treatment of chloroplasts of spinach, however, rather inhibited the activity of succinoxidase (unpublished).

2. Treatment of the chloroplast fraction with lipase brought about considerable solubilization and activation of succinoxidase.

3. Optimum pH of 5.0 for oxidation of succinate by the various fractions was the same as that for the oxidation of the other intermediates of the Krebs cycle.

4. The succinoxidase activity in the particulate fractions was stimulated by addition of cytochrome c and strongly inhibited by azide and cyanide. The enzyme activity in the soluble fraction was affected by the inhibitors to a much less extent.

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摘 要

佐々木喜美子： ベゴニア葉中のコハク酸酸化酵素系についての研究

ベゴニアの葉の磨砕液から分離したか粒区分について、そのコハク酸酸化酵素の性質についてしらべた。

1) コハク酸、フマル酸、リンゴ酸、および α -ケトグルタル酸はか粒区分によって酸化され、その活性はか粒区分を ageing することによりかなり上昇した。

2) か粒区分をリパーゼで処理することにより、コハク酸酸化酵素はかなり溶出し、かつその活性の上昇が認められた。

3) コハク酸、その他の Krebs cycle に属する有機酸の酸化のさいの最適 pH は 5.0 付近であった。

4) 各種区分によるコハク酸の酸化は cytochrome c の酸化によって促進された。

5) Cyanide, azide はか粒区分によるコハク酸の酸化を著しく、また溶出区分による酸化をかなり弱く阻害した。(北海道大学理学部植物学教室)