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CO_2 Evolution in Peroxisomes Isolated from Spinach Leaves at Various Ages

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시금치 잎의 葉齡에 따라 분리한 Peroxisome 에서의 CO_2 放出

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ABSTRACT

The rates of photorespiration and total CO_2 fixation depending on leaf ages of spinach (*Spinacia oleracea* L.) were investigated. Metabolic rates of glycolate and glyoxylate in isolated peroxisomes were also measured. The rate of photorespiration and total CO_2 fixation ability increased with the maturing of leaf, but decreased with senescence. Activities of enzymes involved in the peroxisomal photorespiratory pathway such as catalase, glycolate oxidase, NADH-glyoxylate reductase and glutamate-glyoxylate transaminase were highest in the mature leaf, but also decreased with aging of leaf. Glutamate-glyoxylate transaminase activity significantly decreased with senescence, especially. The metabolic rate of glycolate was observed to be lower than that of glyoxylate in isolated peroxisomes. Glycolate seemed to be metabolized mainly to glycine, however, it also oxidized to CO_2 when glycolate was supplied as a substrate for glycine synthesis instead of glyoxylate. The conversion rates of glycolate and glyxylate into CO_2 increased with the senescence of leaves.

INTRODUCTION

In C_3-plant, a large amount of newly fixed CO_2 is released by a process known as photorespiration (Tolbert, 1971; Kisaki *et al.*, 1971; Kelly *et al.*, 1976), and the carboxyl carbon of glycolate is claimed to be the main source of this photorespiratory CO_2 (Zelitch, 1965; Goldworthy, 1970; Jackson and Volk, 1970). The glycolate is metabolized to glyoxylate by glycolate oxidase and glyoxylate is converted into glycine by the amination reaction. The enzymes responsible for amination of glyoxylate are serine; glyoxylate aminotransferase and glutamate; glyoxylate aminotransferase (Tolbert *et al.*, 1968; Carpe and Simith, 1974; Walton

and Butt, 1981; Betsche, 1983; Yu *et al.*, 1984). Glyoxylate, however, could be oxidized to CO_2 and formate by the action of hydrogen peroxide or superoxide free radicals in peroxisome (Zelitch, 1972b; Halliwell and Butt, 1974). Alpha carbon of glyoxylate as well as carboxyl carbon is also broken down to produce CO_2 by the reaction with the H_2O_2 (Leek *et al.*, 1972; Halliwell, 1974; Grodzinski, 1979). But the other many reports have shown that peroxisomal catalase reduced the hydrogen peroxide generated during glycolate oxidation, so that photorespiratory CO_2 evolution occurred from the conversion of glycine to serine in mitochondria (Tolbert, 1980; Ogren, 1984). On the other hand, Larsen *et al.* (1981) showed that in photosynthesizing cells, added ammonia decreased the carbon in carbohydrates from carbon dioxide fixation and increased in carboxylic acids and amino acids. Moreover, amination of glyoxylate was restricted on the inhibition of glutamine synthetase by L-methionine-sulfoximine (Lee and Lee, 1985). Those reports indicated that the site and amount of CO_2 evolution could be changed by the available pool size of amino acids, especially by that of glutamate.

In this study, therefore, the alteration of the rate and site of photorespiratory CO_2 evolution with respect to leaf age is examined to investigate the relationship between photorespiratory carbon and amino acids metabolism.

MATERIALS AND METHODS

Plant material. Spinach (*Spinacia oleracea* L.) was grown in a greenhouse. The first foliage leaf was used as material. Samples were harvested on the interval of three weeks.

Isolation of peroxisome. Samples were washed with distilled water and the mid vein was removed. About 100 g of leaves were homogenized with 200 ml of glycylglycine buffer (pH 7.5) containting 0.5 M sucrose for 3 seconds in a homogenizer. The homogenate was filtered through four layers of muslin and then centrifuged at 500×g for 25 min. The supernatant was further centrifuged at 6,000×g for 20 min. The precipitates were gently resuspended with homogenizing medium for density gradient centrifugation.

Density gradient centrifugation. A step gradient was made with 3 ml of 2.5 M, 5 ml of 2.3 M, 6 ml of 1.8 M, 7 ml of 1.5 M and 6 ml of 1.3 M sucrose soluton containing 20 mM glycylglycine (pH 7.5), respectively. 3 ml of prepared sample was applied to the top of the gradient and then centrifuged at 41,000×g for 5 hrs. After centrifugation, fractions were collected from the bottom of the tube by piercing with a needle (Halliwell, 1974). The peroxisomal fraction was identified by the assay of glycolate oxidase activity (De Jong, 1973). All these operations were carried out below 4℃.

Enzyme assay. Catalase (EC. 1. 11.1.6.) was assayed by the method of Lück (1963) measuring the decomposition of hydrogen peroxide spectrophotometrically.

Glutamate-glyoxylate aminotransferase (EC.2.6.1.4) activities were assayed by the formation of glycine-1-^{14}C form glyoxylate-1-^{14}C (0.02 μCi, 20 μmole) following the method of Kisaki

and Tolbert (1969) and glyoxylate reductase (EC.1.1.1.26) was assayed by measuring the oxidation of NADH at 340 μm (Zelitch, 1955).

Measurement of metabolic rates of glycolate and glyoxylate. The release of $^{14}CO_2$ from glycolate-1-^{14}C and glyoxylate-1-^{14}C was measured as described by Grodzinski and Butt (1976). 100 μl of peroxisomal fraction was incubated with glycolate-1-^{14}C (0.25 μCi, 5 mM), 0.33 mM FMN and 33 mM glycylgycine (pH 8.0) in a total 3 ml at 25 C for 2 hrs. Glyoxylate was used also at a concentration of 5 mM (0.25 μCi). $^{14}CO_2$ released from glycolate or glyoxylate was collected in 0.2 ml of 20% (w/v) KOH and counted with the liquid scintillation counter. Glycine-1-^{14}C synthesized form glycolate or glyoxylate was purified by the ion exchanger (Dowex 50w H^+ type) of the chromatography method (Zelitch, 1972a) and counted with the liquid scintillation counter.

Measurement of photorespiration. Photorespiration of leaf discs was measured by the method of Zelitch (1968). Leaf discs were cut out with the cork borer and 6 discs (approximately 120 mg) were floated on 2 ml water in a CO_2 fixing vessel. $^{14}CO_2$ was liberated from $NaH^{14}CO_3$ (2 μCi, 10 μmole) and assimilated into leaf discs for 50 min under 180 μmol quanta min · sec. Excess $^{14}CO_2$ was removed and $^{14}CO_2$ released from the leaf discs under CO_2-free air was collected with the monoethanolamine: ethoxyethanol=2:1 (v/v) solution. Photorespiratory rates were calculated from the amout of $^{14}CO_2$ released between 10 and 30 min.

RESULTS AND DISCUSSION

The patterns of photorespiration and total $^{14}CO_2$ fixation of three stages in leaf age are given in Table 1. In immature leaves, the total amount of $^{14}CO_2$ released from the leaf under light condition was 7×10^4 cpm/mg fr.wt. and 6.03×10^4 cmp/mg fr.wt. under dark condition. The light/dark ratio was, then, measured as 1.23. In mature leaves the amount of $^{14}CO_2$ evolution was 9.21×10^4 in light and 4.57×10^4 cpm/mg fr. wt. in dark, but in senescent leaves the amount was 8.48×10^4 nd 5.18×10^4 cpm/mg fr.wt., respectively. Typical C_3-plant is known to exhibit a light/dark ratio of CO_2 evolution greater than 3. The greater the light/dark is, the higher the photorespiratory activity is (Zelitch, 1968; Kennedy, 1976). In our experiment, the rate of photorespiration was highest in mature leaves as the light/dark ratio was 2.01. This suggests that photorespiratory activity of leaves coincides with the change of photosyntheic activity according to age (Table 1).

Activities of four enzymes in each stage of leaf development are given in Table 2. As maturing progressed, all four enzyme activities were observed to increase and activities of all enzymes to decrease with senescence. But the catalase activitiy remained higher compared with the other enzymes. Catalase is known to exist at a characteristic level which is less affected by the development or senescence of leaves (Breidenbach, 1976). On the other hand, NADH-glyoxylate reductase and glutamate-glyoxylate aminotransferase significantly decreased with the aging of leaves, whereas the rate of decrease of glycolate oxidase activity was lower than of the

first two enzymes. In the photorespiratory pathway, glycolate oxidase is an irreversible enzyme and the enzyme activity is inhibited by glycolate accumulates in large amounts (Tolbert, 1980).

Lee and Lee (1984, 1985) showed that the rate of glycine formation in peroxisomes might be affected by the free amino acid pool size in pea leaves. Therefore, the significant decrease of glutamate; glyoxylate aminotransferase activity seemed to be caused not only by the diminution of glyoxylate, but by the decrease of available pool size of gluramate as well.

The metabolic rate of glycolate in peroxisomes of mature leaves was much higher than that of immature or senescent leaves (Table 3). The amount of CO_2 released from glycolate was 1.04 μmoles/mg protein h in peroxisomes isolated from mature leaves, whereas that of senescent leaves was 0.47 μmoles/mg protein h. Synthesis of glycine from glycolate was also highest in mature leaves. But the ratio of CO_2 formation to glycine synthesis in senescent leaves was much higher than that in mature leaves. These results showed the tendency of glycolate to metabolize to CO_2 rather than to glycine with the progress of senescence. Metabolic rates of glyoxylate in peroxisomes were shown in Table 4. It was characteristic that almost all of glyoxylate converted into glycine, so the ratio of CO_2/glycine formation from glyoxylate was much lower than that from glycolate. This phenomenon may be explained by the fact that since the production of hydrogen peroxide is catalyzed by the glycolate oxidase, addition of glycolate to peroxisome stimulates the formation of hydrogen peroxide (Lorimer and Andrews, 1981). Thus the probability of oxidation of glyoxylate by hydrogen peroxide

Table 1. Comparison of photorespiratory $^{14}CO_2$ evolution in three stages of leaf development under light and dark conditions

Leaf stage	Total $^{14}CO_2$ fixed	$^{14}CO_2$ evolution under light	$^{14}CO_2$ evolution in dark	Light/Dark
Immature	16.83×10^6	7.40×10^4	6.03×10^4	1.23
Mature	39.91×10^6	9.21×10^4	4.57×10^4	2.01
Senescent	11.69×10^6	8.48×10^4	5.18×10^4	1.64

Instantly after measuring photorespiration, the leaf discs were extracted with 80% ethyl alcohol and distilled water to determine the total CO_2 fixation (cpm/mg fr.wt.).

Table 2. Activities of enzymes involved in the peroxisomal photorespiratory pathway in each stage of leaf development (nmoles of substrate conversion/mg protein. min)

Leaf stage	Catalase	Glycolate oxidase	NADH-glyoxylate reductase	Gultamte-glyoxylate aminotransferase
Immature	1410×10^3(1.5)*	895(2.1)*	1.01(2.9)*	2260(1.3)*
Mature	1447×10^3(1.5)	1021(2.4)	1.34(3.8)	5880(3.0)
Senescent	945×10^3(1)	424(1)	0.35(1)	1790(1)

( )* indicate the relative values compared with senescent stage.

may increase with the treatment of glycolate to peroxisomes. From our results, we suggested that glycolate and glyoxylate were chiefly metablized to glycine in mature spinach leaves, but with aging, peroxisomal CO_2 evolution increased with the decrease of metabolic rate of glyoxylate into glycine as shown in Fig. 1.

Table 3. Conversion of glycolate into CO_2 and glycine in isolated peroxisomes

Leaf stage	Metabolic rate of glycolate (% of total)	CO_2 evolution (μ moles/mg protein h)	Glycine formation (μ moles/mg protein h)	CO_2/Glycine (percent)
Immature	6.48(3.9)*	0.87(1.9)*	18.71(2.5)*	4.6
Mature	12.28(7.4)	1.04(2.2)	28.97(3.9)	3.6
Senescent	1.66(1)	0.47(1)	7.51(1)	6.3

( )* indicate the relative values compared with the senescent stage.

Table 4. Conversion of glyoxylate into CO_2 and glycine in isolated peroxisome

Leaf stage	Metabolic rate of glyoxylate (% of total)	CO_2 evolution (μ moles/mg protein h)	Glycine formation (μ moles/mg protein h)	CO_2/Glycine (percent)
Immature	49.39(2.2)*	1.51(2.8)*	126.37(2.1)*	1.2
Mature	62.29(92.8)	0.19(0.4)	311.27(5.1)	0.06
Senescent	22.15(1)	0.55(1)	60.96(1)	0.9

( )* indicate the relative values compared with the senescent stage.

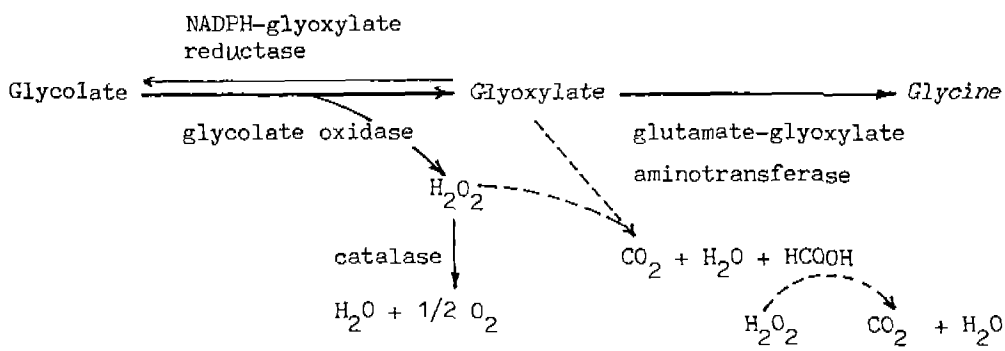

Fig. 1. Scheme for metabolic pathway of glycolate and glyoxylate in peroxisomes. Solid lines indicate the major pathway in mature leaves. Dotted lines indicate the alteration of metabolism according to senescence.

摘 要

시금치(*Spinacia oleracea* L.)잎을 사용하여 나이에 따라 光呼吸率과 CO_2 固定能을 측정하였고, 分離된 peroxisome에서 glycolate와 glyoxylate의 代謝率 및 光呼吸과정에 관련된 catalase, glycolate oxidase, NADH-glyoxylate reductase 그리고 glutamate-glyoxylate transaminase등의 活性을 측정하였다.

光呼吸率과 CO_2 固定能은 잎이 성숙함에 따라 증가하였으며 노화함에 따라 감소하였다. 光呼吸 과정에 관여하는 catalase, glycolate oxidase, NADH-glyoxylate reductase 그리고 glutamate-glyoxylate transaminase 등의 活性은 모두 성숙한 잎에서 가장 높았으며 노화가 진행됨에 따라 그 활성이 감소하였으며, 특히 glytamate-glyoxylate transaminase의 활성이 가장 현저한 감소현상을 보였다. 分離된 peroxisome에서 glycolate의 代謝率은 glyoxylate에 비해 현저히 낮았으며, 대부분은 glycine으로 전환되었다. 그러나 glycolate와 glyoxylate를 각각 투여한 경우 glycolate가 CO_2로 전환되는 率이 높았으며, 이러한 경향은 잎이 노화함에 따라 증가하였다.

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