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# Influence of Benomyl on Photosynthetic Capacity in Soybean Leaves

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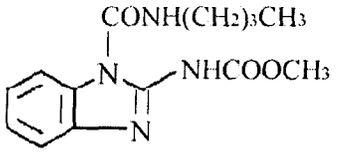
**Abstract** This investigation was performed to study the influence of benomyl on photosynthetic pigments and enzymes in soybean leaves. Chlorophyll and pheophytin levels were reduced by benomyl 45 days after greening. These results indicate that chlorophyll *a* and *b*, and pheophytin must be controlled by benomyl. SDS-PAGE analysis showed that 50 and 14.5 kD polypeptides represented as the large and small subunits of rubisco. In the both of these subunits, the band intensity of the control was significantly higher than that after benomyl treatment, indicating that these two subunits are affected by benomyl. Benomyl strongly inhibited both the activity and content of rubisco as its concentration was gradually increased. However, it remains unclear whether this reduction of rubisco level was due to a reduced level of rubisco activase. Two major polypeptides of 46 and 42 kD were identified as rubisco activase subunits by SDS-PAGE. The intensity of these two bands was shown to be higher in the control than after benomyl treatment. These results indicate that the rubisco decrease resulting from increased benomyl concentrations was caused by rubisco activase. A significant decrease in both the activity and content of rubisco activase by benomyl was also observed. These results suggest that the decrease in rubisco level caused by benomyl is accompanied by a decrease in both the activity and content of rubisco activase.

*Keywords*: benomyl, chlorophyll, rubisco, rubisco activase, soybean

## INTRODUCTION

Benomyl (methyl[1-(butylcarbamoyl)-1*H*-benzimiazol-2-yl] carbamate, Scheme 1), a systemic benzimidazole fungicide, is one of the most widely used for the control of a variety of plant diseases [1-4], and one of the most effective fungicides used in mycorrhizal research, as it is believed to be a compound with very low phytotoxicity [5-7]. Because of its instability, residues of benomyl in crops are determined by acid hydrolysis of benomyl to the stable compound, carbendazim, which is then analyzed by HPLC [8].




Scheme 1. Structure of benomyl.

Benomyl inhibits both the formation of the cytoskeleton and the nuclear division by inhibiting the formation of microtubules [9]. Benomyl in MS medium also reduces culture contaminants singnificantly [10]. Aragaki *et al.* [11] found that benomyl inhibits the yield and the root length of cucumber. The effect of benomyl on fungal phosphate transport and fungal enzyme activity in the cucumber *Glomus* symbiosis was reported by Hale and Sanders [12], and Thingstrup and Rosendahl [13], respectively.

Rubisco (EC 4.1.1.39) catalyzes two reactions, namely, the carboxylation [14] and the oxygenation [15] of RuBP. The former reaction involves $CO_2$ fixation in the photosynthetic carbon reduction cycle and the latter 2-phosphoglycolate production in the photorespiration pathway. The enzyme requires prior activation with $CO_2$ and $Mg^{2+}$ to catalyze both reactions [16,17].

Rubisco activase promotes the activation of rubisco [18,19] in the presence of ATP and RuBP [20], and is known to be present in large concentrations in the leaves of green plants. It is composed of two polypeptides of approximately 41 kD and 44 kD in most plant species, including, *Arabidopsis thaliana* wild type, spinach, tobacco, soybean, kidney bean, pea, celery, oat and barley. In maize leaves, however, immunoblot analysis indicates the presence of only the smaller polypeptide [21]. Both rubisco activase polypeptides were absent from an *Arabidopsis rca* mutant, which required an elevated $CO_2$ concentration for growth [18].

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However, the effects of benomyl on the photosynthetic pigment and enzymes are unknown. The objectives of this study were (i) to determine the effects of benomyl on chlorophyll and pheophytin; (ii) to determine the effect of benomyl on rubisco by analyzing the peptides profiles using SDS-PAGE, by measuring its activity using an ATP dependent hydrolysis assay, and by determining the content of rubisco by spectrophotometric assay; and (iii) to determine the activity and content of rubisco activase in order to clarify whether the effects of benomyl on rubisco are correlated with rubisco activase level.

## MATERIALS AND METHODS

### Plant Culture

Seeds of soybean (*Glycine max* L.) were germinated and grown in a growth chamber as described by Roh *et al.* [22,23].

### Chlorophyll and Pheophytin Determination

Chlorophyll and pheophytin contents were determined as described by Inskeep and Bloom [24], and Vernon [25], respectively. For the chlorophyll and pheophytin estimation in leaves, the leaves were frozen in liquid nitrogen, and ground to a fine powder, extracted with DMF and 80% acetone in the dark, respectively, and centrifuged for 5 min at 8,000 x *g*. Chlorophyll was measured spectrophotometrically using its specific absorption coefficients at 664.5 nm and 647 nm. Absorbance readings for pheophytin were taken at 655 and 666 nm. The following equations were used to give the concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll and pheophytin.

$$\text{Chlorophyll } a \text{ (mg/g fr. wt.)} = 12.70\ A_{664.5} - 2.79\ A_{647}$$

$$\text{Chlorophyll } b \text{ (mg/g fr. wt.)} = 20.70\ A_{647} - 4.62\ A_{664.5}$$

$$\text{Total chlorophyll (mg/g fr. wt.)} = 17.90\ A_{647} + 8.08\ A_{664.5}$$

$$\text{Pheophytin } (\mu g/mL) = 6.75\ A_{666} + 26.03\ A_{655}$$

### Purification of Rubisco and Rubisco Activase

Rubisco and rubisco activase were purified from soybean leaves using a modification of the method described by Wang *et al.* [26]. Frozen leaf powder ground in liquid nitrogen was added to 50 mM BTP (pH 7.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.01 mM leupeptin, 1.5% PVPP and 3 mM MBT, and stirred until the ice melted. The solution filtered through cheesecloth and Miracloth was centrifuged for 40 min at 30,000 × *g*. $(NH_4)_2SO_4$ powder was slowly added to the supernatant to 35% saturation and stirred for 30 min. The supernatant was collected by centrifugation for 10 min at 8,000 x *g* and brought to 55% of $(NH_4)_2SO_4$ saturation by adding the powder. The pellet was collected by centrifuging for 10 min at 8,000 × *g* resuspended in buffer A [50 mM Tricine (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 10 mM DTT and 2 mM MBT], and 50% PEG-10,000 was added to a final concentration of 18%. The resulting precipitate was collected by centrifuging for 10 min at 8,000 × *g* and resuspended in buffer A. Resuspended solution was then loaded onto a Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. The column was washed with the same buffer containing 0.1 M NaCl before starting elution with a linear gradient from 0.1 to 0.5 M NaCl at a flow rate of 1 mL/min. 3 mL fractions with high rubisco activity were pooled.

The 35% $(NH_4)_2SO_4$ pellet collected by centrifugation for 10 min at 8,000 × *g*, as described above, was resuspended in buffer B [20 mM BTP (pH 7.0), 0.2 mM ATP, 10 mM $MgCl_2$ and 2 mM MBT]. 50% (w/v) PEG-10,000 was added to the resuspended pellet to a concentration of 18%, stirred for 5 min, and centrifuged for 10 min at 8,000 × *g*. The pellet was then dissolved in buffer B. The supernatants were collected by spinning for 10 min at 20,000 × *g* and were loaded onto a Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0). The column was eluted with 20 mM BTP (pH 7.0) before continuing with a linear gradient from 0 to 0.5 M NaCl in 20 mM BTP (pH 7.0) at a flow rate of 1 mL/min. Only a single fraction (3 mL) with the highest rubisco activase activity was collected.

### Assay of Rubisco and Rubisco Activase Activity

Rubisco activity was determined at 25°C using the method described by Racker [27]. Rubisco assay solution contained, in a final volume of 1 mL, 0.45 mL of $H_2O$, 0.05 mL of 1 M Tris buffer (pH 7.8), 0.02 mL of 0.006 M NADH, 0.05 mL of 0.1 M GSH, 0.05 mL of 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.02 mL of 0.025 M 3-phosphoglycerate kinase, 0.05 mL of 0.05% α-glycerophosphate dehydrogenase-triose phosphate isomerase, 0.02 mL of 0.025 M ribulose bisphosphate, 0.06 mL of 0.2 M ATP, 0.02 mL of 0.5 M $MgCl_2$, 0.15 mL of 0.5 M $KHCO_3$ and 0.06 mL of the enzyme solution to be tested. Oxidation of NADH was monitored at 340 nm during the conversion of 3-phosphoglycerate to glycerol 3-phosphate. One unit was defined as the amount which catalyzes the cleavage of 1 μM of ribulose bisphosphate per min.

Rubisco activase activity was determined using the ATP hydrolysis method of Robinson and Portis [28]. The rate ADP appearance was measured by determining the decreased absorption at 340 nm using the reaction mixture [50 mM Tricine (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM phospho*enol*pyruvate, 0.3 mM NADH, 40 units/mL pyruvate kinase, and 40 units/mL lactate dehydrogenase]. The reaction was started by adding rubisco activase. One unit was defined as the amount required to hydrolyze 1 μM of ATP per min.

### Content Determination of Rubisco and Rubisco Activase

Rubisco protein was determined spectrophotometrically as $A_{280} \times 0.61$ = mg/mL [29]. Rubisco activase protein was determined using a Bio-Rad protein assay kit (Bio-Rad Lab. Richmond, CA, USA), based on the Bradford method [30] using BSA as a standard.

### Electrophoresis

SDS-PAGE was carried out following the procedure described by Laemmli [31] using 13% gels. Gels were stained with Coomassie blue to visualize proteins.

## RESULTS AND DISCUSSION

### Photosynthetic Pigments

Chlorophyll *a* is located in the reaction center of the photosystem, and chlorophyll *b* is mainly located in the light-chlorophyll-harvesting complex, rather than the reaction center [32,33]. To assess the contribution of benomyl to the regulation of photosynthesis, we first examined the effects of benomyl on chlorophyll and pheophytin. Changes of the chlorophyll *a* and *b* contents, the chlorophyll *a*/*b* ratio and total chlorophyll content during the greening of leaves in soybean treated with benomyl are shown in Table 1. The data in Table 1 shows that when the benomyl concentration was raised from 0 ppm to 15 ppm, leaf chlorophyll *a* and *b* content, the chlorophyll *a*/*b* ratio and total chlorophyll content all decreased 45 days after greening. Camp *et al.* [34] reported that the changes in chlorophyll content closely paralleled the changes in photosynthesis using wheat leaves. Quick *et al.* [35] suggested that the content of chlorophyll was affected by rubisco.

Pheophytin, the primary electron acceptor in PSII, is a modified chlorophyll a molecule in which the central $Mg^{2+}$ is replaced by two hydrogen atoms [36]. Changes of the pheophytin content during the greening of leaves in soybean treated with benomyl are shown in Table 2. Pheophytin contents at control, 5, 10 and 15 ppm at 45 days were 0.368, 0.346, 0.338 and 0.303 μg/mL, respectively. Increased benomyl concentration during greening decreased the pheophytin content in control and in the benomyl treatments, and this continued to decrease as greening progressed (data not shown). These results indicate that chlorophyll *a* and *b*, and pheophytin are controlled by benomyl.

### Rubisco

The effects of benomyl on rubisco were investigated by analyzing the SDS-PAGE peptide profiles and measuring the activity and level of rubisco. The SDS-PAGE patterns of rubisco purified from soybean leaves is shown in Fig. 1, which show two major polypeptides at 50 and 14.5 kD identified as the large and small subunits of rubisco. This result is consistent with that previously reported for rubisco by SDS-PAGE [22] and Western blotting [23] showed two polypeptides at 50 and 14.5 kD. In the cases of both the 14.5 and 50 kD subunits, the intensity of the control was significantly higher than that after benomyl treatment. These results indicate that both subunits of rubisco are affected by benomyl treatment.

Benomyl inhibits arbuscular mycorrhizal (AM) fungal enzyme activities. Kough *et al.* [37] and Sukarno *et al.* [6] found that benomyl reduced the activity of AM fungal succinate dehydrogenase, and Thingstrup and Rosendahl [13] showed that the alkaline phosphatase and malate dehydrogenase activities of *Glomus intraradices* were also inhibited by benomyl.

As shown in Fig. 2, 11.39 of rubisco activity in the control decreased to 9.46 at 5 ppm, 8.73 at 10 ppm and 7.27 units/mL at 15 ppm of benomyl. Benomyl strongly inhibited rubisco activity as its concentration gradually was increased. The results from this study indicate that increasing benomyl levels during growth affects the

Table 1. Changes of the chlorophyll *a* and *b* content, chlorophyll *a*/*b* ratio and total chlorophyll content during greening in soybean treated with benomyl

| Benomyl concentration (ppm) | Chlorophyll content (mg/g fr. wt.)* | |
|---|---|---|
| 0 | Chl. *a* | 0.696 |
| 5 | | 0.638 |
| 10 | | 0.570 |
| 15 | | 0.529 |
| 0 | Chl. *b* | 0.231 |
| 5 | | 0.212 |
| 10 | | 0.205 |
| 15 | | 0.193 |
| 0 | Chl. *a*/*b* | 3.017 |
| 5 | | 3.007 |
| 10 | | 2.782 |
| 15 | | 2.738 |
| 0 | Total Chl. | 0.927 |
| 5 | | 0.851 |
| 10 | | 0.774 |
| 15 | | 0.722 |

* Data at 45 days after greening

Table 2. Change of the pheophytin content during greening in soybean treated with benomyl

| Benomyl concentration (ppm) | Pheophytin content * (μg/mL) |
|---|---|
| 0 | 0.368 |
| 5 | 0.346 |
| 10 | 0.338 |
| 15 | 0.303 |

* Data at 45 days after greening

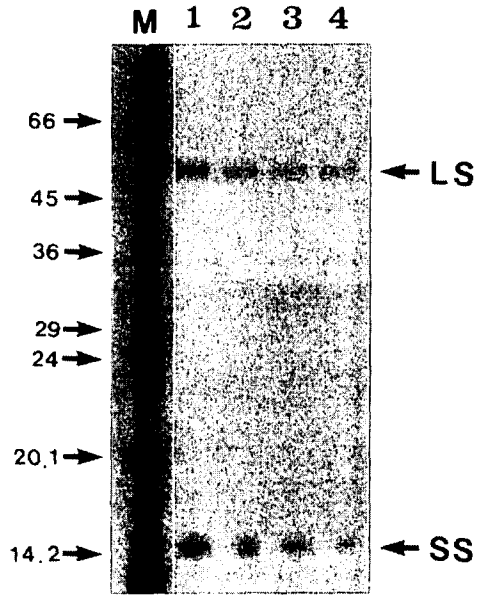


Fig. 1. SDS-PAGE detection of rubisco purified from soybean leaves. Proteins (20 μg) were separated on 13% SDS-PAGE gels. M, molecular weight standards; lane 1, no treatment; lane 2, 5 ppm; lane 3, 10 ppm; lane 4, 15 ppm of benomyl. Rubisco subunits indicated by an arrow.

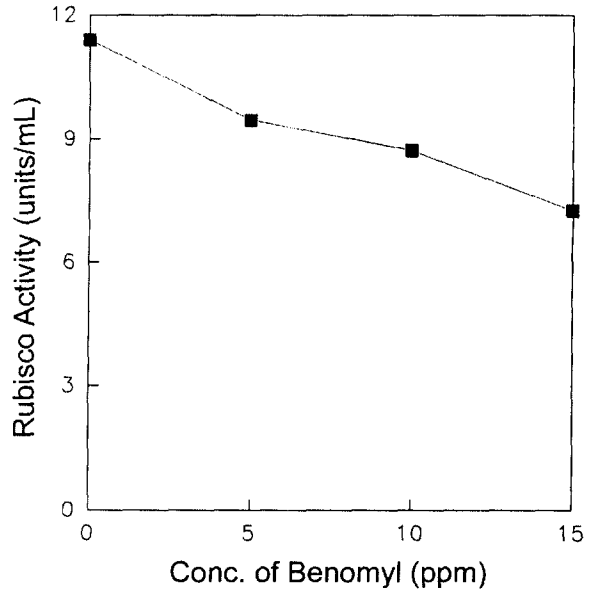


Fig. 2. Effects of benomyl on the activity of rubisco in soybean leaves.

rubisco activity of soybean leaves. The inhibitory effect of benomyl on rubisco activity observed in this experiment is in agreement with the results of Kough *et al.* [37] and Sukarno *et al.* [6] and Thingstrup and Rosendahl [13].

Downton *et al.* [38] reported that the reduction in

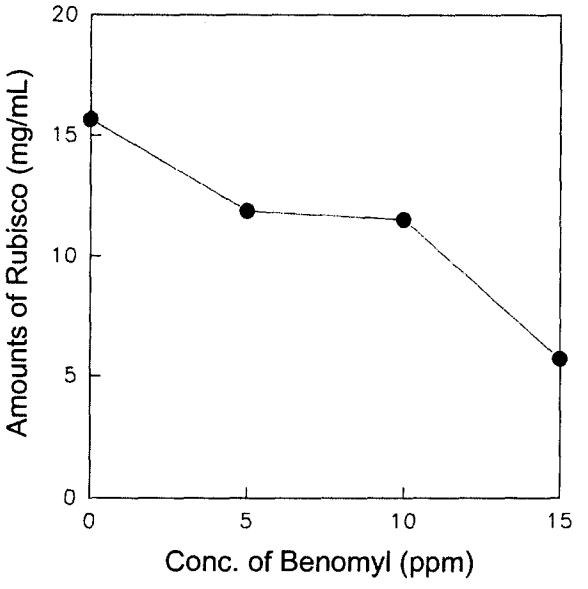


Fig. 3. Effects of benomyl on the content of rubisco in soybean leaves.

rubisco activity was associated with a reduced amount of rubisco protein. Makino *et al.* [39] examined the relationship between rubisco protein and its activity in rice leaves, and as a result, reported that the change in the carboxylase activity was caused by a change in the level of the enzyme protein. Under the assumption that a decrease in the rubisco activity as induced by benomyl is related with the rubisco level, we determined the rubisco content in leaves grown at various benomyl concentrations. As shown in Fig. 3, Rubisco content rapidly decreased with each benomyl treatment according to the increased benomyl concentration: add was 15.68 in the control, 11.90 at 5 ppm, 11.53 at 10 ppm and 5.73 mg/mL at 15 ppm benomyl, respectively. These results suggest that decreased rubisco activity caused by benomyl is related with the amount of rubisco.

## Rubisco Activase

Rubisco levels were promoted by the carbamylation of rubisco activase in the presence of RuBP [20] by the controlled release of RuBP from the active site of rubisco, in a process requiring the hydrolysis of ATP [40-42]. The question remains as to whether the inhibitory effects of benomyl on rubisco activity and content are correlated with rubisco activase or not. To answer this question, we determined the activity and content of rubisco activase using the SDS-PAGE band pattern. As shown in Fig. 4, bands of rubisco activase analyzed by SDS-PAGE were idetified as two polypeptides at 46 kD and 42 kD. These results were similar to these obtained during the immunological detection of rubisco activase polypeptide from leaf extracts of soybean [21] and kidney bean [23], and from barley rubisco activase

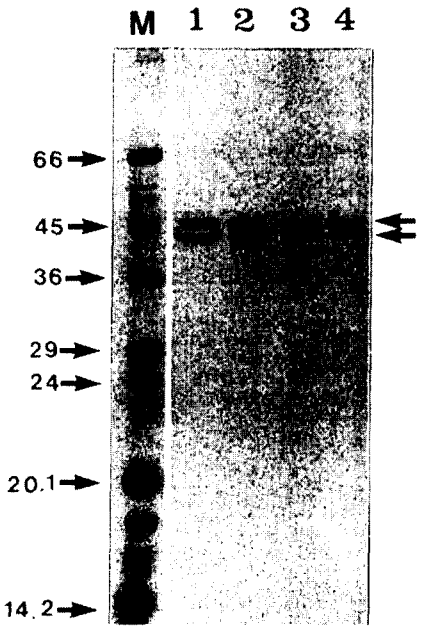


Fig. 4. SDS-PAGE detection of rubisco activase purified from soybean leaves. Proteins (20 μg) were separated on 13% SDS-PAGE gels. M, molecular weight standards; lane 1, no treatment; lane 2, 5 ppm; lane 3, 10 ppm; lane 4, 15 ppm of benomyl. Rubisco activase indicated by an arrow.

expressed in transformant *E. coli* [43] separated by SDS-PAGE. Remarkable differences in the densities of both the 46 kD and 42 kD bands were found between the control and the benomyl treatments.

The assay for rubisco activase activity is based on its ability to catalyze the hydrolysis of ATP [19], while rubisco activase contents were measured by spectrophotometric assay [29]. The changes in the activity and content of rubisco activase after increasing benomyl concentrations are shown in Fig. 5 and 6, respectively. The activity and content of rubisco activase was induced by benomyl: from 15.1 to 11.3 units/mL in activity; and from 0.473 to 0.333 mg/mL in content. Rubisco activase activity and content showed patterns of change which were similar to those of rubisco. These results suggest that a decrease in rubisco levels by benomyl is accompanied by a decrease in both the activity and the content of rubisco activase.

## CONCLUSION

Based on these experiments collectively, it is believed that benomyl negatively effects the pigments and enzymes involved in photosynthesis, that temporal sterilization by benomyl causes a decrease in photosynthetic production

In future studies concerning the effect of benomyl on rubisco, to verify the relationships between benomyl, rubisco and rubisco activase, we intend to focus on carbamylation rate measurements at various benomyl concentrations by using a dual beam spectrophotometer [44].

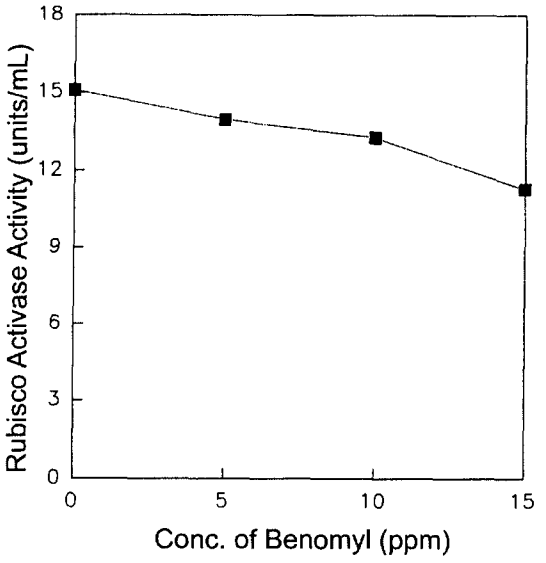


Fig. 5. Effects of benomyl on the activity of rubisco activase in soybean leaves.

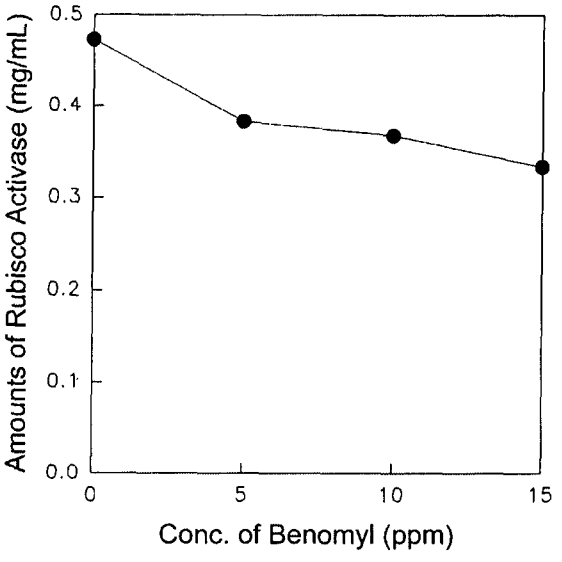


Fig. 6. Effects of benomyl on the content of rubisco activase in soybean leaves.

## NOMENCLATURE

BTP : Bis-tris propane
DMF : *N,N*-Dimethylformamide
DTT : Dithiothreitol
GSH : Glutathione
MBT : Mercaptobenzothiazole
PEG : Polyethylene glycol

PMSF : Phenylmethylsulfonylfluoride
PSII : Photosystem II
PVPP : Polyvinylpolypyrrolidone

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