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Sucrose Regulates Growth and Activation of Rubisco in Tobacco Leaves *In Vitro*

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Abstract The influence of sucrose on *in vitro* growth, chlorophyll content, and rubisco/rubisco activase were studied in tobacco leaves. The most pronounced effect on *in vitro* growth and the chlorophyll content was found at 4% sucrose. The rubisco content increased with increasing concentrations of sucrose, but a point was reached beyond which the increasing concentrations of sucrose caused an inhibition of this enzyme. The rubisco activity showed patterns of change similar to the rubisco content. These data suggest that sucrose may have an affect on the activation and induction of rubisco and that sucrose can be both a positive effector and negative effector depend on its concentration. The degree of intensity of 55 and 15 kD polypeptides, which were identified as the large and small subunit of rubisco, respectively, by SDS-PAGE analysis at 4% sucrose was significantly higher than that of other treatments, indicating that sucrose had an effect on both subunits. We subsequently examined whether the rubisco content and activity of being induced by sucrose is associated with rubisco activase. The rubisco activase content at 4% sucrose was higher than that of other treatments. A similar change pattern was also observed in the activity of rubisco activase. The intensity of two 52 and 51 kD polypeptide bands at 4% sucrose was higher than that of corresponding bands of other treatments. The stimulatory and inhibitory effects of rubisco by sucrose seemed to be caused by rubisco activase.

Keywords: chlorophyll, *in vitro,* rubisco, rubisco activase, sucrose, tobacco

INTRODUCTION

Disaccharide sucrose is a principal end product of carbon fixation during the photosynthetic reaction in many higher plants. It is the form in which most fixed organic carbons are translocated from photosynthetically active source tissue to heterotrophic sink organs, such as developing leaves, the shoot apex, roots and reproductive organs, via phloem tissue [1]. Excess sucrose accumulates in the leaves, but predominantly in storage tissues. Sucrose is mostly stored in the vacuole which comprises close to 70% of the cell volume. The concentration of sucrose in the leaf vacuole at peak light periods reached 11 mM. In contrast, the cytosol which accounts for only 28% of the cell volume accumulated almost 55 mM sucrose [2]. The stored sucrose not only provides a substrate for energy and synthesis of cell matter and other storage glycosides such as starch and fructans, but also mobilizes and utilizes during germination and growth of the developing plant.

Sucrose is also transported into the hypocotyl, and sucrose accumulation enhances anthocyanin production in the hypocotyl [3]. It regulates amino acid biosynthesis in storage tissues such as potato tubers [4]. Mizukami *et al.* reported that a high sucrose concentration is necessary to give a high yield of shikonin derivatives in *Lithospermum erythrorhizon* callus [5].

Sucrose is also the most used carbon source in heterotrophic and mixotrophic tissue culture. Exogenous sucrose negatively affected *in vitro* growth of *Solanum tuberosum* [6] and photosynthesis of *in vitro* plantlets of *Gardenia jasmoides* [7]. Conversely, stimulating effects of sugars on growth and photosynthesis have been reported for *in vitro* plantlets of tobacco [8], potato [9], and avocado [10]. In spite of the considerable literature on this subject, however, little is known about the effect of sucrose on the photosynthesis at the rubisco activation level by rubisco activase *in vitro*.

In order to investigate the effect of sucrose, whether it is positive or negative, we studied the influence of exogenous sucrose on growth and photosynthesis base on chlorophyll and rubisco/rubisco activase level in leaves of tobacco induced from the stem of seedling *in vitro.*

MATERIALS AND METHODS

In Vitro Culture of Tobacco Seedlings

Tobacco (*Nicotiana tabacum* L.) seeds were germinated and grown aseptically in a cell culture vessel containing MS agar medium [11]. The shoots were cut into

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2 cm segments and used as explants. The explants were placed on an induction MS medium supplemented with 0-5% sucrose after the pre-subculture stage on sucrose free medium. These explants were maintained on these media at 27°C ± 2°C under light for 16 h (800 μM m^{-2} s^{-1} PFD) and dark photoperiod for 8 h [12]. Fully expanded leaves from mature plants at 7 weeks were used as a the specimen in this study. All experiments were independently triplicated.

Determination of Chlorophyll Content

Chlorophyll contents were measured spectrophotometrically according to the method described by Inskeep and Bloom [13]. The leaves from each treatment were extracted with dimethylformamide in the dark and centrifuged for 5 min at 8,000 × *g*. The following equations were used for the calculation of chlorophyll *a*, chlorophyll *b*, and total chlorophyll from the supernatants.

Chlorophyll *a* (mg/g fw) = 12.70 $A_{664.5}$ - 2.79 A_{647}
Chlorophyll *b* (mg/g fw) = 20.70 A_{647} - 4.62 $A_{664.5}$
Total chlorophyll (mg/g fw) = 17.90 A_{647} + 8.08 $A_{664.5}$

Purification of Rubisco and Rubisco Activase

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and rubisco activase were purified from tobacco leaves [12, 14]. Frozen leaf tissue was pulverized in a mortar under liquid nitrogen and then extracted in buffer containing 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. The leaf slurry was filtered through four layers of cheesecloth and one layer of Miracloth. This filtered solution was centrifuged at 30,000 × *g* for 40 min. $(NH_4)_2SO_4$ powder was slowly added into the supernatant to a saturation level of 35%, and it was stirred for 30 min. The supernatant and pellet were collected by centrifugation at 8,000 × *g* for 10 min.

The supernatant was brought to 55% saturation by the addition of $(NH_4)_2SO_4$ powder. The pellet collected by centrifugation at 8,000 × *g* for 10 min was resuspended in 20 mM BTP (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$ and 2 mM MBT (buffer A), and 50% PEG-10K was added to give a final concentration of 18%. The resulting precipitate was collected by centrifugation at 8,000 × *g* for 10 min and resuspended in buffer A. This solution was loaded onto a Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. Then, the column was washed with the same buffer containing 0.1 M NaCl before the start of the elution with a linear gradient from 0.1 to 0.5 M NaCl at a flow rate of 1 mL/min. 3 mL fractions were pooled, and assayed for rubisco content and activity.

To purify rubisco activase in the resuspended pellet obtained above, 50% (w/v) PEG-10K was added to buffer A to give a final concentration to 18%, and this solution was centrifuged at 8,000 × *g* for 10 min. The pellet was again dissolved in buffer A, and then this solution was cleared by spinning at 20,000 × *g* for 10 min. This process of dissolving and spinning was performed one more time. The collected supernatants were loaded onto a 20 mL Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0). The column was eluted with 20 mM BTP (pH 7.0) at a flow rate of 1 mL/min before continuing with a linear gradient from 0 to 0.5 M NaCl in 20 mM BTP (pH 7.0). 3 mL fractions were pooled, and assayed for rubisco activase content and activity.

All purification processes were performed at 4°C except as indicated.

ELISA

In order to coat of the antigen, 100 μL of various dilutions of two enzymes (rubisco and rubisco activase) in 0.1 M sodium carbonate-bicarbonate coating buffer (pH 9.5) was added to each well of microplate. After overnight incubation at room temperature, the plate was washed with 0.01 M PBS (pH 7.4) containing 0.05% Tween 20. To eliminate nonspecific binding, 250 μL of 0.1% BSA in 0.01 M PBS (pH 7.4) was added to each well and incubated for 1 h at 37°C. After washing and adding of 50 μL of various dilutions of two enzymes in 0.01 M PBS (pH 7.4), 50 μL of different dilutions of a rabbit anti-rubisco and anti-rubisco activase antiserum was added to each well as the primary antibody [15], and mixture was incubated for 30 min at 37°C. The plate was again washed as described above, and then, 100 μL of peroxidase-conjugated goat anti-rabbit IgG diluted to a ratio of 1:20,000 in 0.01 M PBS (pH 7.4) containing 0.1% BSA was added and incubated for 30 min at 37°C. The plate was washed as previously described and 100 μL of peroxidase substrate (OPD tablets in 10 mL of 0.05 M citrate/0.1 M sodium phosphate buffer (pH 5.0) containing 30% of H_2O_2) was added. After incubation at RT in the dark for 20 min, the reaction was terminated by addition of 0.1 mL of 1 N HCl. Absorbance at 490 nm was determined by an ELISA microplate reader (Bio-Rad Model 3550-UV).

Activity Assays

Rubisco activity was determined spectrophotometrically at 25°C by monitoring NADH oxidation at 340 nm [16]. The assay medium contained 1 M Tris buffer (pH 7.8), 0.006 M NADH, 0.1 M GSH, 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.025 M 3-phosphoglycerate kinase, 0.05% α-glycerophosphate dehydrogenase-triose phosphate isomerase, 0.025 M RuBP, 0.2 M ATP, 0.5 M $MgCl_2$, 0.5 M $KHCO_3$, and purified rubisco solution in a final volume of 1 mL. One unit of enzyme was defined as the amount of enzyme producing 1 μM of RuBP per min.

Rubisco activase activity was determined as the ability to produce ADP in an ATP-dependent reaction with the absorption at 340 nm [17]. The purified rubisco activase solution was added to a volume of 0.4 mL of the activation reaction mixture, which contained 50 mM Tricine

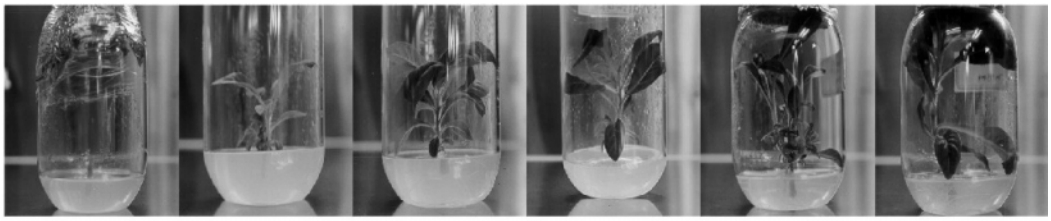

Fig. 1. Effect of sucrose on the growth of tobacco plants *in vitro*.

Table 1. Effect of sucrose concentration on the chlorophyll content in tobacco leaves Plants were grown for 7 weeks

Sucrose (%)	*Chl. a*	*Chl. b*	*Chl. a/b*	Total chl
		(mg/g fw)		
1	13.81	22.12	0.62	35.93
2	14.11	22.23	0.63	36.34
3	14.12	22.35	0.63	36.47
4	14.25	22.52	0.63	36.77
5	13.71	22.08	0.62	35.79

(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. One unit was defined as the amount that catalyzed the cleavage of 1 μM ATP per min.

SDS-PAGE

Electrophoresis was performed in a 20% polyacrylamide gel at room temperature by the method of Laemmli [18]. The protein samples (40 μg) were solubilized at 100°C for 10 min before being loaded on the gel. After electrophoresis, proteins were stained by Coomassie Brilliant Blue R-250, and then destained by 7.5% acetic acid.

RESULTS AND DISCUSSION

In Vitro Growth of Tobacco

Sucrose concentration can affect the growth rate and the yield of secondary metabolite in plant cell cultures [19]. In this study, tobacco plants not grown on medium without sucrose (Fig. 1), indicating that *in vitro* plant was in heterotrophic culture, because of insufficient light intensity. These results were in agreement with those of Schnapp and Preece [20] who showed that lowering the sucrose level to 0 resulted in fewer and shorter axillary shoots and reduced the height of carnation plant grown *in vitro*. When sucrose concentrations in MS medium were increased from 1 to 5%, after 7 weeks of treatment, the medium containing 4% sucrose produced longer and more vigorous shoots and roots than the same medium containing varying concentrations of sucrose (Fig. 1). These results indicate that sucrose had a marked effect on the growth of tobacco under the given environment. Although the maximum growth was achieved at 4% sucrose, these results were consistent with that of Park *et al*. [21] who showed that 3% sucrose enhanced cell growth, and the relative cell growth is lower in MS medium supplemented with 5 and 7% sucrose in a *Ginko biloba* cell culture. Tomato microplants grown in the medium with 5 g/L sucrose had less shoot and root growth than those with 10, 20, or 30 g/L sucrose [20]. Similar to our results, Kim *et al*. [22] also reported that the formation of a somatic embryo induced from the leaf callus of *Lycium chinense* Mill was increased at 4% sucrose. In contrast to our results, however, Zhang *et al*. [23] observed the maxmum cell growth at 3% sucrose in suspension cultures of *Panax notoginseng*.

Chlorophyll

Chlorophylls are pigments capable of absorbing visible radiation that will initiate the photochemical reactions of photosynthesis in higher plants. Chlorophyll *a* is located in the reaction center of the photosystem, and chlorophyll *b* is located in the light-chlorophyll-harvesting complex, rather than the reaction center [24,25].

To assess the contribution of sucrose to the regulation of photosynthesis, the changes of the chlorophyll *a* and *b* contents, the chlorophyll *a*/*b* ratio and total chlorophyll content in the leaves of tobacco treated with 1-5% sucrose concentrations were examined (Table 1). When the sucrose concentration was raised from 1 to 5%, the most pronounced effect on leaf chlorophyll *a* content was found to be at 4% sucrose after 7 weeks of treatment. A

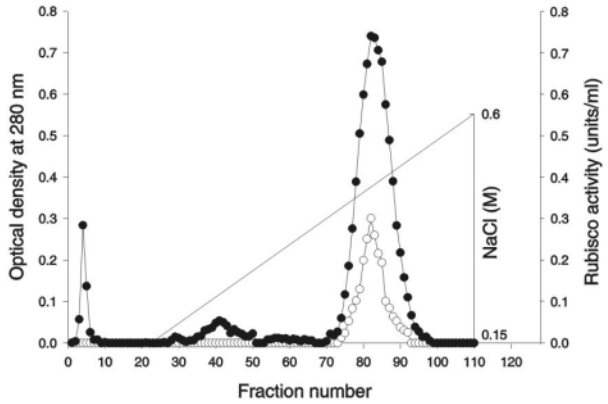

Fig. 2. Elution profile for protein (●) and activity (○) of rubisco from anion exchange chromatography on Q-Sepharose column. Rubisco was purified from tobacco leaves with 4% sucrose. Its activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0.15-0.6 M NaCl gradient in 20 mM BTP (pH 7.2).

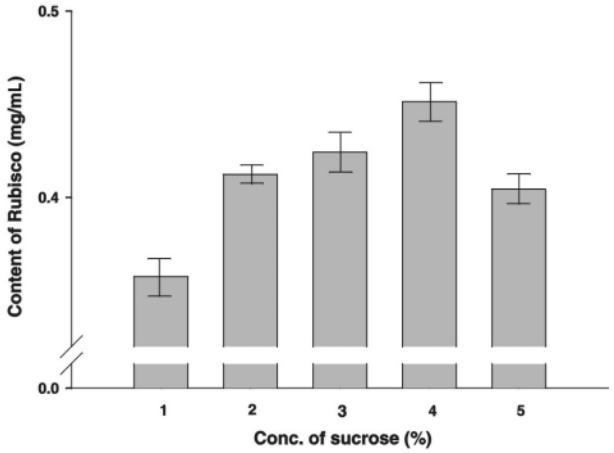

Fig. 3. Effect of sucrose on the content of rubisco in tobacco leaves. Plants were grown on MS medium that included sucrose ranging from 1 to 5%.

similar effect of sucrose was observed on chlorophyll *b*. However, a similar change pattern was not observed in the chlorophyll *a*/*b* ratio. Total chlorophyll content followed the same trend as the content of chlorophyll *a* and *b*. These results indicate that chlorophyll *a* and *b* are controlled by sucrose.

Camp *et al*. [26] reported that the changes in chlorophyll content closely parallel the changes in photosynthesis of wheat leaves. Quick *et al*. [27] suggested that the content of chlorophyll is affected by rubisco.

Rubisco and Rubisco Activase

The rubisco was assembled in a catalytically inactive form and was activated by the binding of activator CO_2 and Mg^{2+} to ε-amino group of Lys-201 within the active site on the large subunit [28].

For the effect of sucrose on rubisco, rubisco was purified from leaves of tobacco grown at various concentrations of sucrose by anion exchange chromatography on Q-Sepharose column (Fig. 2). And then its content and activity were detected by an ELISA and through the oxidation of NADH, respectively.

The rubisco content increased with increasing concentrations of sucrose, but a point (4%) was reached beyond which increasing concentrations of sucrose caused an inhibition of this enzyme (Fig. 3). The rubisco content was substantially decreased at the highest sucrose supply and was significantly greater when there was a small concentration of sucrose in the avocado leaves grown *in vitro* [10]. A decreased rubisco content is often correlated with a decreased photosynthetic rate and also with an increased concentration of sugars in cell cultures [29]. This correlation is also observed in other experimental systems in which sugars accumulate in leaves [30].

The reduction in rubisco activity can be associated with a reduced amount of rubisco protein [31]. Rubisco activity showed patterns of change similar to the rubisco content (Fig. 4). The activity was highest when the concentration of sucrose was at 4%, but it was significantly lower when it was at and below 3% and at 5%. It was found that 4% sucrose best stimulated the content and activity of rubisco, whereas high sucrose concentration acted in an inhibitory manner. These results indicate that sucrose may affect the activation and induction of rubisco and that sucrose can be both a positive and negative effector depending on its concentration.

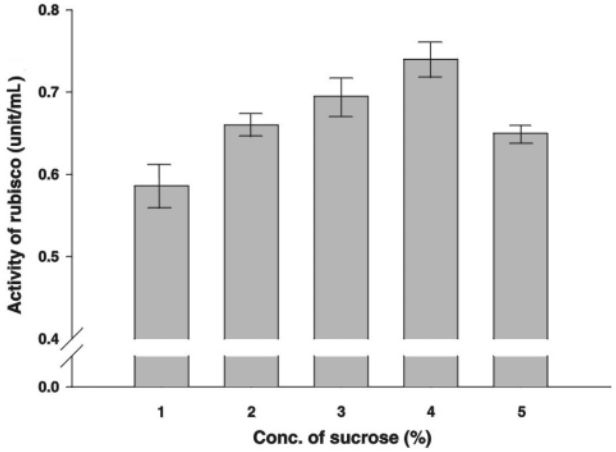

Fig. 4. Effect of sucrose on the activity of rubisco in tobacco leaves. Plants were grown on MS medium that included sucrose ranging from 1 to 5%.

The increased maximum photosynthetic rate *in vitro*, which was observed when sucrose is decreased, suggests that the exogenous carbohydrate supply inhibits development of the photosynthetic system and photosynthetic rate [32], specifically by decreasing rubisco activity [33].

In order to verify the degree of regulation by sucrose, the intensity of 55 and 15 kD rubisco proteins in each of the sucrose treatments, identified as the large and small

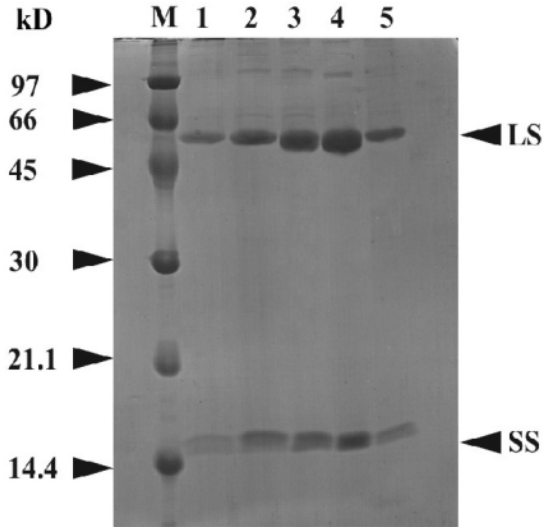

Fig. 5. SDS-PAGE analysis of rubisco purified from tobacco leaves. Proteins were (40 μg) separated on 20% SDS-PAGE gels. M, molecular weight standards; lane 1, 1% sucrose; lane 2, 2% sucrose; lane 3, 3% sucrose; lane 4, 4% sucrose; lane 5, 5% sucrose. Protein was stained with Coomassie blue. LS, large subunit; SS, small subunit.

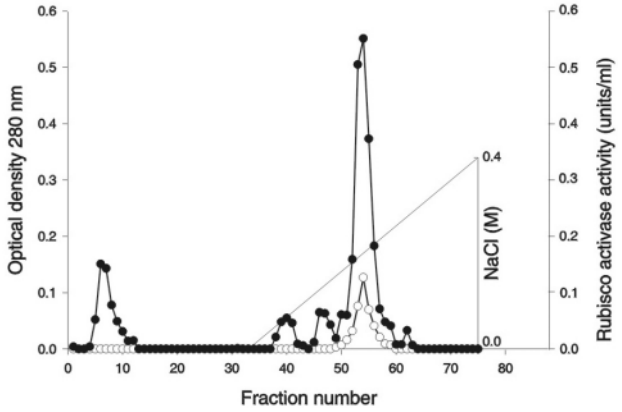

Fig. 6. Elution profile for protein (●) and activity (○) of rubisco activase from anion exchange chromatography on Q-Sepharose column. Rubisco activase was purified from tobacco leaves with 4% sucrose. Its activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0-0.4 M NaCl gradient in 20 mM BTP (pH 7.0).

subunit of rubisco, was confirmed by SDS-PAGE (Fig. 5). Unlike our result, however, two subunits, at 50 and 14.5 kD, had been recognized in leaves of kidney bean [34], soybean [35], and jackbean [12]. The intensity level of two rubisco subunits detected at 4% sucrose was significantly higher than that with other sucrose treatments. These results suggest that both subunits are affected by sucrose.

Rubisco activase is a new type of chaerone, which functions to promote and maintain the catalytic activity of

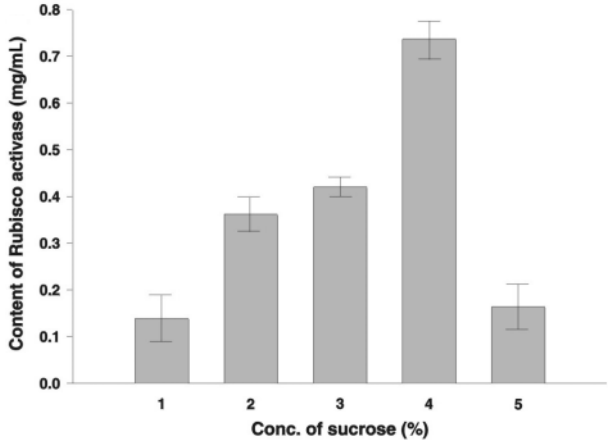

Fig. 7. Effect of sucrose on the content of rubisco activase in tobacco leaves. Plants were grown on MS medium that included sucrose ranging from 1 to 5%.

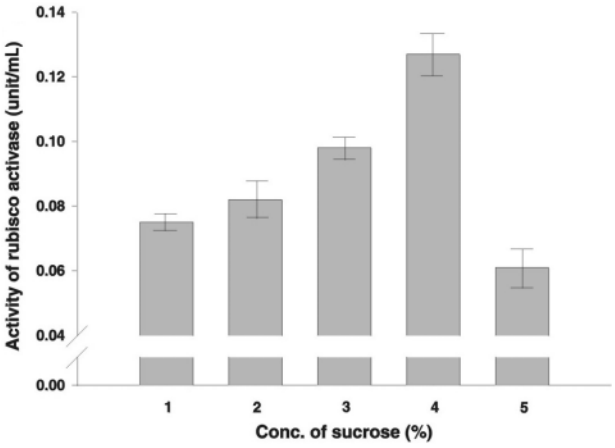

Fig. 8. Effect of sucrose on the activity of rubisco activase in tobacco leaves. Plants were grown on MS medium that included sucrose ranging from 1 to 5%.

rubisco [36] in the presence of ATP and RuBP [37,38].

Under the assumption that the effect of sucrose on rubisco content and activity may be associated with rubisco activase, this enzyme was purified on a Q-Sepharose column (Fig. 6). The content of rubisco activase was calculated using absorbance determined by an ELISA, and its activity was measured in the presence of ATP [17].

Like the result of the rubisco content, sucrose strongly increased the content of rubisco activase as its concentration was gradually increased to 4%, while it was significantly decreased when it was at 5% (Fig. 7). The same pattern of sucrose was also confirmed in terms of the activity of rubisco activase (Fig. 8). The finding that rubisco activase was remarkably increased upon 4% sucrose may clarify the observed sucrose response of rubisco activase.

The intensity of two 52 and 51 kD polypeptide bands

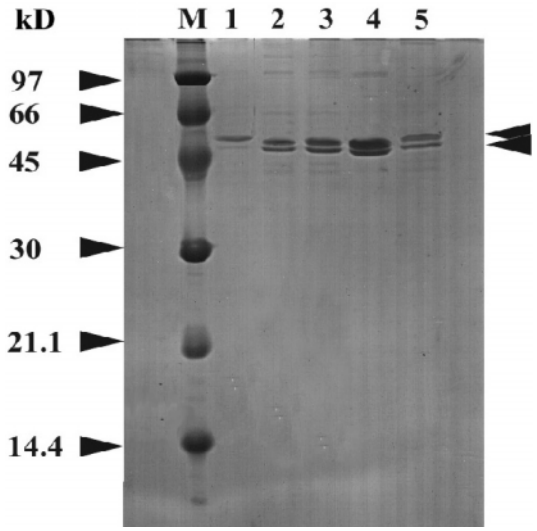

Fig. 9. SDS-PAGE analysis of rubisco activase purified from tobacco leaves. Proteins were (40 μg) separated on 20% SDS-PAGE gels. M, molecular weight standards; lane 1, 1% sucrose; lane 2, 2% sucrose; lane 3, 3% sucrose; lane 4, 4% sucrose; lane 5, 5% sucrose. Protein was stained with Coomassie blue. Rubisco activase is indicated by an arrow.

at 4% sucrose was higher than that of the corresponding bands from the other treatments (Fig. 9). These results indicate that sucrose concentration affect rubisco activase. The result presented here was consistent with reports showing spinach rubisco activase, which was synthesized from a cloned cDNA in *E. coli,* being capable of activating rubisco in an ATP-dependent reaction *in vitro* [39]. Two major polypeptides were independently capable of catalyzing rubisco activation *in vitro* [40].

Based on the collective results of rubisco and rubisco activase, in conclusion, the stimulatory and inhibitory effects of rubisco by sucrose seem to be caused by rubisco activase.

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