Isolation and Partial Characterization of Homogeneous Ferredoxin-sulfite Reductase from a Red Alga, *Porphyra yezoensis*

Osamu KOGUCHI and Goro TAMURA*

Department of Agricultural Chemistry, Faculty of Horticulture,
Chiba University, Matsudo, Chiba 271, Japan

Received January 20, 1989

Ferredoxin-dependent sulfite reductase (Fd-SiR) (EC 1.8.7.1) was purified about 1136-fold, with a yield of 11%, from fresh thalli of *Porphyra yezoensis* by a procedure involving ammonium sulfate precipitation, DEAE-cellulose chromatography, Butyl-Toyopearl chromatography, Sephadex G-100 gel filtration and ferredoxin-Sepharose affinity chromatography. The purified enzyme was apparently homogeneous, as judged on polyacrylamide disc gel electrophoresis, with a specific activity of 100 units/mg of protein. The molecular weight of the enzyme was estimated to be 70 kilodaltons by gel filtration. On subunit analysis by SDS-PAGE, a single band corresponding to molecular weight of 65 kilodaltons appeared. The purified enzyme (Fd-SiR) showed 5-times higher ferredoxin-dependent activity than methyl viologen-linked activity. In the oxidized form, the enzyme exhibited absorption maxima at 278, 390 (Soret band), 586 (α band) and 714 (CT band) nm, indicating that siroheme is involved in the catalysis of sulfite reduction. The absorbance ratios, $A_{390}:A_{278}$ and $A_{586}:A_{390}$, were 0.32 and 0.31, respectively. A plot of the substrate (sulfite) and electron donor (ferredoxin) concentrations versus enzymatic (Fd-SiR) activity yielded sigmoidal curves, giving Hill coefficients ($\bar{n}$) of 2.3 (for sulfite) and 2.7 (for ferredoxin), respectively. Antibody against the isolated enzyme was raised in rabbits. Analysis of the antiserum by immunodiffusion suggested that it was specific against isolated Fd-SiR. Using the antiserum, dot immunoblotting was performed to determine the immunological similarity of Fd-SiRs from *Porphyra yezoensis*, *Spirulina platensis*, *Brassica chinensis* and *Spinacia oleracea*. The tests revealed that the four forms of assimilatory Fd-SiR have antigenic determinants in common.

Sulfite reduction, observed in photosynthetic algal cells, is generally considered to be the final step of assimilatory sulfate reduction, in which sulfite is reduced to the level of sulfide according to the following equation.^{1~6)}

This reaction step is catalyzed by the enzyme, sulfite reductase (SiR).^{1,2,5)} Some information on a red algal (*Porphyra yezoensis*) SiR has been obtained through experiments with an artificial dye, reduced methyl viologen (MV), as an electron donor.^{4,5)}

Recently, however, photoreduced ferre-

doxin (Fd) was reported to be a physiological electron donor in a cyanobacterial (formerly blue-green algal) (*Spirulina platensis*) sulfite reductase system.⁷⁾ Although MV-SiR (hydrogen-sulfite: (acceptor) oxidoreductase, EC 1.8.99.1) of *Porphyra yezoensis* has been highly purified and extensively characterized, the purified enzyme has been demonstrated to be unable to couple with reduced ferredoxin.⁸⁾ In addition to this MV-SiR, we wish to report the purification of a ferredoxin-dependent SiR (Fd-SiR) (hydrogen sulfite: ferredoxin oxidoreductase, EC 1.8.7.1) from a red alga, *Porphyra yezoensis*.

Abbreviations: SiR, sulfite reductase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Fd, ferredoxin; TKG-100 buffer, 50 mM Tris-H₂SO₄ buffer pH 7.8, containing 10% glycerol and 100 mM K₂SO₄; CT band, charge transfer band.

* To whom reprint requests should be addressed.

Materials and Methods

Materials. Fresh thalli of *Porphyra yezoensis*, grown in Tokyo Bay in the winter, were kindly supplied by Mr. Araki of the Yamamoto Nori Research Laboratory. *Porphyra* and spinach ferredoxins were purified by the method of Tagawa and Arnon⁹⁾ ($A_{422} : A_{277} = 0.45$). Ferredoxin-Sepharose was prepared by the method of Shin and Oshino.¹⁰⁾ 5-Deazariboflavin was prepared by the method of Yoneda.¹¹⁾

The following chemicals were purchased from commercial sources: DEAE-cellulose (DE-52) (Whatman Ltd); Sephadex G-100 (Pharmacia Fine Chemicals); Coomassie brilliant blue G 250 (Fluka AG); a calibration protein kit (ovalbumin, bovine serum albumin, aldolase, catalase and ferritin) (Boehringer Mannheim); and a SDS-PAGE calibration protein kit (phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α -lactalbumin) (Pharmacia Fine Chemicals). Toyopearl HW-55 and Butyl Toyopearl 650s were kindly supplied by TOSOH Mfg. Co. Other chemicals were of analytical grade.

Assaying Fd- and MV-SiR activities. The assay method for Fd-SiR activity was the same as that described previously.^{7,12)} One unit of Fd-SiR activity was defined as the amount of enzyme which caused the oxidation of 1 μ mol of reduced ferredoxin per min under the assay conditions described. MV-SiR activity was measured spectrophotometrically at 25°C under anaerobic conditions in a Thumberg-type cuvette (light path, 1 cm). The standard assay mixture contained 50 mM Tris-H₂SO₄ buffer, pH 7.8, 2 mM EDTA, 0.03 mM 5-deazariboflavin, 0.2 mM methylviologen, 50 μ l of an appropriately diluted enzyme preparation and 1 mM sodium sulfite, in a total volume of 2 ml. The absorption at 604 nm of the solution in a cuvette was recorded and then the cuvette was illuminated with a PRR-500-W lamp placed 5 cm away from it. After incubation for 2 min, the reaction was started by adding sodium sulfite from the side arm and the change in absorbance at 604 nm was followed. One unit of MV-SiR activity was defined as the amount of enzyme which caused the oxidation of 1 μ mol of reduced methyl viologen per min under the assay conditions described.

Physical and chemical measurements. Spectrophotometric determinations were performed with a Hitachi U-3200 and a Hitachi 556 double beam spectrophotometer using 1 cm light-path cuvettes. Molecular weight estimation by SDS-PAGE was performed by the method of Laemmli.¹³⁾ Analytical PAGE was performed by the method of Ornstein and Davis.¹⁴⁾ The amino acid composition was determined with a high sensitivity amino acid analyzer (Hitachi 835 Amino Acid Analyzer) after a sample had been hydrolyzed with 6 N HCl for 24 hr, 48 hr, or 72 hr at 100 °C in an evacuated sealed tube. The protein content was determined by the method of Bradford¹⁵⁾ using bovine serum albumin as a standard.

Preparation of antiserum against Fd-SiR. Homogeneous *Porphyra* Fd-SiR (about 1 mg) emulsified with complete Freund's adjuvant (Difco Laboratories) was administered subcutaneously to a New Zealand white rabbit. Booster injections were given fourth at two week intervals, with the same amount of antigen. At two weeks after the final injection, whole blood was obtained. This allowed to stand at 37°C for 1 hr and then at 4°C overnight, and then the serum was obtained by centrifugation. Serum from an unimmunized rabbit was used in the control experiment. The antiserum was used without further purification.

Dot immunoblotting analysis. The Fd-SiR solution was spotted onto dry Biotex nylon membrane paper strips (2.5 × 5 cm) with a Drummond microdispenser. The peroxidase activity on the sheet was detected by the method of Nakane.¹⁶⁾

Results

Enzyme purification

All the purification procedures were carried out in a cold room maintained at 0~4°C. Unless otherwise stated, dialysis was performed overnight against the indicated buffer.

Step 1. Preparation of a crude extract. *Porphyra* thalli were washed several times with tap water to free them from salts and then, with the addition of several drops of toluene, subjected to autolysis in two volumes of 0.2 M Tris-HCl buffer, pH 7.7, for 96 hr at 4°C. The autolysate thus obtained was filtered through cheesecloth with the aid of a hand press and the filtrate was used as the crude extract. One kilogram (wet weight) of thalli was used in this experiment.

Step 2. Acrinolum fractionation. To the above crude extract, a 1% acrinolum solution was added slowly with stirring to 0.04%. The mixture was stirred for an additional 10 min and the precipitate was removed by filtration through a layer of absorbent cotton. To the filtrate obtained above were added 2-mercaptoethanol, phenylmethylsulfonyl fluoride and polyclar AT to final concentrations of 10 mM, 0.5 mM and 5%, respectively. The resulting solution was filtered through cheesecloth.

Step 3. Ammonium sulfate fractionation. Solid ammonium sulfate was slowly added to the filtrate from the preceding step (750 ml) to obtain 30% saturation. After removing

the resulting precipitate by centrifugation ($10,000 \times g$ for 20 min), ammonium sulfate was further added to the supernatant to final 85% saturation. The precipitate formed was collected by centrifugation ($10,000 \times g$ for 30 min), suspended in buffer A (50 mM Tris-HCl buffer, pH 7.7, containing 100 mM NaCl and 50 mM K_2SO_4) and then dialyzed against the same buffer.

Step 4. Column chromatography on DEAE-cellulose (DE-52). The dialyzed solution (90 ml) was applied to a DE-52 column (5×25 cm) previously equilibrated with buffer

B (20 mM potassium phosphate buffer, pH 7.5, containing 5 mM K_2SO_4 and 200 mM NaCl) at the flow rate of 2 ml per min and 20 ml fractions were collected. The elution pattern is shown in Fig. 1. The active fractions (62~72) on column chromatography on DE-52 were pooled.

Step 5. Column chromatography on Butyl-Toyopearl 650s. The pooled fractions (210 ml), described above, were supplemented with ammonium sulfate to 35% saturation. The resulting solution was applied on a Butyl-Toyopearl 650s column (3×35 cm) previously equilib-

Fig. 1. Elution Profile of *Porphyra* Ferredoxin-sulfite Reductase on a DEAE-cellulose Column.

○—○, enzyme activity in units per ml; ●—●, amount of protein in milligrams per ml.

Fig. 2. Elution Pattern of *Porphyra* Fd-SiR on a Butyl Toyopearl Column.

○—○, Fd-SiR activity; ●—●, protein.

rated with buffer B 35% saturated with ammonium sulfate. After the column had been washed with the same buffer, a linear concentration gradient of the eluent was established with 500 ml of equilibrating buffer in the mixing vessel and 500 ml of buffer B in the reservoir. Elution was performed at the flow rate of 0.8 ml per min and 10 ml fractions were collected. A typical chromatographic pattern obtained is shown in Fig. 2. The eluates in tubes 32~47 in Fig. 2 were combined and concentrated in a dialysis sac against buffer B

75% saturated with ammonium sulfate. The precipitate formed in the dialysis sac was collected by centrifugation at $18,000 \times g$ for 10 min and then dissolved in a minimal amount of buffer C (20 mM potassium phosphate buffer, pH 7.5, containing 150 mM K_2SO_4).

Step 6. Gel filtration on Sephadex G-100. The enzyme solution obtained above (3 ml) was filtered through a Sephadex G-100 column (2×90 cm) equilibrated with buffer C. Elution was performed with the same buffer (flow rate,

Fig. 3. Sephadex G-100 Gel Filtration.

○—○, Fd-SiR activity; ●—●, protein.

Fig. 4. Ferredoxin-Sepharose Affinity Chromatography.

○—○, Fd-SiR activity; ●—●, protein. The arrow indicates the point at which buffer D was replaced by buffer C.

0.3 ml/min) and 2 ml fractions were collected. Figure 3 shows the elution pattern. The active fractions (65~73) were combined, concentrated with Sephadex G-100 powder and then dialyzed against buffer D (30 mM Tris-H₂SO₄ buffer, pH 7.8, containing 1 mM K₂SO₄).

Step 7. Column chromatography on ferredoxin-Sepharose. The dialyzed enzyme solution (5 ml) was diluted 2 times with deionized distilled water and then applied to a column of ferredoxin-Sepharose 4B (2 × 2 cm) previously equilibrated with buffer D. Undesired proteins were removed from the column by washing with the equilibrating buffer and Fd-SiR was eluted afterwards with buffer C (Fig. 4).

The fractions showing specific activities of

higher than 95 units/mg of protein were pooled and concentrated with Sephadex G-100 powder, and used as the isolated enzyme in the subsequent experiment.

Table I is a summary of the purification, starting from 1 kg of *Porphyra thalli*. In this experiment the final preparation represented about 1136-fold purification over the ammonium sulfate fraction and the recovery was 11%.

Properties of the purified enzyme

(1) *Purity of the enzyme.* When the isolated enzyme was subjected to disc PAGE, a single intensely stained protein band was observed (Fig. 5a).

Table I. PURIFICATION OF Fd-SiR FROM *Porphyra yezoensis*

Fraction	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Yield (%)
(NH ₄) ₂ SO ₄ (30~85%) ^a	175	2000	0.088	100
DEAE-cellulose	125	190	0.66	71
Butyl-Toyopearl	90	18	5.0	51
Sephadex G-100	50	2.9	17.0	29
Ferredoxin-Sepharose ^b	20	0.2	100	11

^a Starting with 1 kg of *Porphyra yezoensis*.

^b Ferredoxin from *Spinacia oleracea*.

Fig. 5. Electrophoresis of the Purified Fd-SiR.

(a) Analytical (10% polyacrylamide) disc gel electrophoresis of about 50 μg of the enzyme preparation. A constant electric current (4 mA per tube) was applied for 2 hr.

(b) SDS slab-PAGE of 2 μg of the purified Fd-SiR on 12% polyacrylamide gel. Proteins were stained with Coomassie brilliant blue G 250. S, Fd-SiR; M, marker proteins.

Fig. 6. Determination of the Molecular Weight of *Porphyra* Fd-SiR.

(a) Determination of the molecular weight of *Porphyra* Fd-SiR by SDS-PAGE. The electrophoretic mobilities (mobilities relative to that of Malachite green) of the marker proteins and Fd-SiR are shown. The marker proteins have the following molecular weights: phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (20,100) and α -lactalbumin (14,400).

(b) Measurement of the molecular weight of Fd-SiR by Toyopearl HW-55 filtration. Fd-SiR (600 μ g/ml) and the marker proteins (2 mg/0.2 ml) were separately filtered on a column of Toyopearl HW-55 (2×100 cm) in TGK-100 buffer. The marker proteins used were catalase (240,000), aldolase (158,000), albumin (45,000) and α -chymotrypsinogen A (25,000).

Fig. 7. Effect of the *Porphyra* Ferredoxin Concentration on the Fd-SiR Activity of *Porphyra* Fd-SiR. The amount of protein used in each assay was 5 μ g. The Hill coefficient ($\bar{n}$) is shown in the inset.

(2) *Molecular weight.* The molecular weight of the isolated Fd-SiR was estimated by SDS-PAGE. The preparation gave a single protein band on SDS-PAGE (Fig. 5b). In Fig. 6a, the R_f values (mobilities relative to Malachite green) for the standard protein's subunits were plotted against the known molecular weights of the subunits. The R_f value for the purified enzyme was 0.38, corresponding to a molecular weight of 65 kilodaltons. Furthermore, the molecular weight of this enzyme in a high ionic strength solution was also determined by gel filtration on a Toyopearl HW-55 column (2×90 cm) equilibrated with TKG-100 buffer. Figure 6b shows a plot of the elution volumes for the marker proteins against their molecular weights. The elution volume of the isolated Fd-SiR was 203 ml, corresponding to 70 kilodaltons.

(3) *Catalytic properties.* The effect of the ferredoxin concentration of Fd-SiR activity of the enzyme is shown in Fig. 7. A plot of ferredoxin concentration *versus* velocity yield-

ed a curve that did not obey the Michaelis-Menten equation. The Hill coefficient ($\bar{n}$) obtained from the Hill plot was 2.70, suggesting the presence of multiple binding sites for ferredoxin on the enzyme molecule, interacting in a positive cooperative manner. The plots of sulfite concentration *versus* velocity also yielded a curve that did not obey the Michaelis-Menten equation. The Hill coefficient ($\bar{n}$) calculated from a Hill plot of the data was 2.30 (Fig. 8).

(4) *Absorption spectrum.* The absorption spectrum of the enzyme in the oxidized form is shown in Fig. 9. Absorption maxima were observed at 278 (protein peak), 390 (Soret band), 586 (α -band) and 714 (CT band) nm. The absorbance ratios 390 to 278 nm and 586 to 278 nm, are 0.32 and 0.10, respectively. Since this absorption spectrum is similar to that reported by Siegel and Davis for the β -subunit of *E. coli* NADPH-sulfite reductase,¹⁷⁾ it can be concluded that the *Porphyra* Fd-SiR molecule contains a siroheme-containing

Fig. 8. Effect of the Sulfite Concentration on the Fd-SiR Activity of *Porphyra* Fd-SiR.

The amount of protein used in each assay was 5 μg .

The Hill coefficient ($\bar{n}$) is shown in the inset.

Fig. 9. Absorption Spectrum of the Purified *Porphyra* Fd-SiR. The enzyme concentration was 600 μg of protein per ml of buffer C.

prosthetic group.

(5) *Amino acid composition.* The results of amino acid analysis of the isolated Fd-SiR are presented in Table II. The purified Fd-SiR sample was extracted from the polyacrylamide gel after electrophoresis in the presence of SDS. The molarity of each amino acid for the Fd-SiR was calculated on the basis of the molecular weight determined by SDS-PAGE.

(6) *Dot immunoblotting analysis.* The results of dot immunoblotting analysis are presented in Fig. 10. The anti-*Porphyra* Fd-SiR reacted with not only *Porphyra* Fd-SiR but also with the enzyme from other photosynthetic organisms, including a filamentous cyanobacterium and two kinds of higher plants, and gave a single precipitation dot. Also, the results suggest that the several Fd-SiRs mentioned above possess antigenic determinants in common and are extremely similar in structure. So it might be concluded that Fd-SiRs from several photosynthetic organisms probably arose from a common ancestor, although they appear to have diverged to a great degree in structural

Fig. 10. Dot Immunoblotting Analysis of Fd-SiRs from Different Sources.

A: Ten microliter drops of the Fd-SiR solutions (300 $\mu\text{g}/\text{ml}$) were spotted on the a dry Biodyne nylon membrane sheet with a Drummond microdispenser.

B: The enzyme solutions (300 $\mu\text{g}/\text{ml}$) supplemented with an amount of one microliter.

features.

Discussion

Methyl viologen-sulfite reductase (MV-SiR) (hydrogen-sulfite: (acceptor) oxidoreductase, EC 1.8.99.1) which can reduce sulfite

Table II. AMINO ACID COMPOSITIONS OF *Porphyra* AND SPINACH Fd-SiRs

Residue	Residues/subunit weight		
	<i>Porphyra</i> Fd-SiR	Spinach 63 K SiR ^a	Spinach 69 K SiR ^a
Asx	63	62	66
Thr ^d	33	31	32
Ser ^d	29	31	32
Glx	63	65	70
Pro	30	32	31
Gly	63	52	52
Ala	68	31	36
Val	34	43	45
Met	16	13	14
Ile	26	29	31
Leu	52	53	59
Tyr	22	21	22
Phe	24	26	26
Lys	35	39	42
His	16	7	8
Arg	37	38	38
Cys	8 ^b	5 ^b	5 ^b
Trp	c	c	c

Details of the hydrolysis method are given under **Materials and Methods**.

^a Data of Krueger and Siegel.²²⁾

^b Measured as cysteic acid after performic acid oxidation.

^c Not determined.

^d Calculated by extrapolation to zero time of hydrolysis.

has been purified from a variety of photosynthetic organisms, including higher plants^{1,2,18)} and algae.^{4,5)}

These enzymes cannot utilize any kinds of physiological electron donor.^{19,20)} More recently, however, we reported the occurrence and extensive purification of plant type (including higher plants and a cyanobacterium) sulfite reductases (Fd-SiRs) (hydrogen-sulfite: ferredoxin oxidoreductase, EC 1.8.7.1), which can utilize reduced ferredoxin as a physiological electron donor.^{7,12,21)}

The present paper described a novel method for purifying Fd-SiR from the thalli of a red alga, *Porphyra yezoensis*. This enzyme, now purified 1136-fold, behaved as an almost homogeneous protein on polyacrylamide gel electrophoresis at pH 8.9 (Fig. 5a). The specific

activity of the purified Fd-SiR (100 μ mol of reduced ferredoxin oxidized per min per milligram of protein) seems to be the highest value reported so far under the assay conditions described. When subjected to the methyl viologen-sulfite reductase assay, the purified Fd-SiR showed lower specific activity (20 μ mol of reduced methyl viologen oxidized per min per milligram of protein).

On SDS-PAGE, the molecular weight of the isolated *Porphyra* Fd-SiR was estimated to be 65 kilodaltons, *i.e.*, about the same as those of enzymes from *Spirulina platensis*⁷⁾ and *Brassica chinensis*.¹²⁾ The molecular weights of Fd-SiRs from *Spinacia oleracea*²¹⁾ and *Spirulina platensis*⁷⁾ so far reported are in the range of 120~136 kilodaltons, as determined on gel filtration under high ionic strength conditions, *i.e.*, with 100 mM K₂SO₄. This suggests that these two enzymes maybe have dimeric forms under these conditions. However, the gel filtration of *Porphyra* Fd-SiR in the presence of 100 mM K₂SO₄ gave a value of 70 kilodaltons, *i.e.*, the same as that of the enzyme from *Brassica chinensis*, indicating the presence of a monomer in this ionic environment. In comparison with these results, the molecular weights of highly purified MV-SiRs so far reported are in the range of 83~85 kilodaltons; Asada *et al.* for spinach leaves and Saito and Tamura for *Porphyra* thalli.^{4,5)}

When the absorption spectrum of *Porphyra* Fd-SiR is compared with that of the β -subunit (a siroheme-containing protein) of NADPH-sulfite reductase from *Escherichia coli*,²²⁾ it can be readily seen that these enzymes are very similar in their chromophoric properties. Thus, the *Porphyra* Fd-SiR exhibits absorption maxima at 278, 390, 586 and 714 nm, which are comparable to the 280, 386, 587 and 714 nm for the β -subunit of the *E. coli* enzyme. This suggests that these two enzymes, together with so far reported Fd-SiRs from other chlorophyllous plants, may have basically similar functional groups, despite their different sources, and further that *Porphyra* Fd-SiR, like the β -subunit of the *E. coli* NADPH-sulfite reductase, has siroheme as its pros-

thetic group. In our previous paper,^{7,12,21)} we reported that Fd-SiRs from *Spinacia*, *Brassica* and *Spirulina* all exhibit absorption maxima at 278~279, 385~388, 585~590 and 714 nm, indicating the presence of siroheme. In contrast to the Fd-SiRs cited, the methyl viologen-linked SiR from *Porphyra yezoensis* thalli reported previously does not contain siroheme.⁵⁾ The absorption spectrum of the *Porphyra* MV-SiR, with a maximum at 385 nm and slight shoulders at 408, 456, 600 and 664 nm, is somewhat different from Fd-SiRs described.

It may be of some interest to point out the difference in amino acid composition between higher plant and algal Fd-SiRs. When compared with the 63- and 69-kilodalton Fd-SiRs from spinach leaves, *Porphyra* Fd-SiR contains significantly greater amounts of Gly, Ala, His and Cys per molecule than the spinach Fd-SiRs. Antiserum against *Porphyra* Fd-SiR was also used to determine immunologically the cross reactivities to other plant Fd-SiRs. Immunoprecipitin dots were obtained between anti-*Porphyra* Fd-SiR and other plant Fd-SiRs. And also it might be concluded that Fd-SiRs from a rather wide range of photosynthetic organisms probably arose from a common ancestor, although they appear to have diverged to a great degree in structural features. Now, it remains to be established whether Fd-SiR and MV-SiR have basically different protein structures or the latter enzyme is an artifact derived from the native Fd-SiR in an early step of the purification.

Acknowledgments. We are indebted to Dr. Sukekatsu Nozaki of Josai University for his guidance in amino acid analysis of Fd-SiRs. We are also grateful to Dr. Kazuo Fukushima of Nihon University School of Dentistry, Matsudo, for his guidance in preparation of the antiserum. We also wish to thank Messrs. Hitoshi Takahashi and

Takaharu Hayakawa for preparation of *Porphyra* ferredoxin and for their assistance in some of the experiments reported in the paper.

References

- 1) G. Tamura, *J. Biochem.*, **57**, 207 (1965).
- 2) K. Asada, G. Tamura and R. S. Bandurski, *J. Biol. Chem.*, **244**, 4904 (1969).
- 3) A. Schmidt and A. Trebst, *Biochim. Biophys. Acta*, **180**, 529 (1969).
- 4) E. Saito, G. Tamura and S. Shinano, *Agric. Biol. Chem.*, **33**, 860 (1969).
- 5) E. Saito and G. Tamura, *Agric. Biol. Chem.*, **35**, 491 (1971).
- 6) A. Schmidt and J. D. Schwenn, Abstracts of Papers, 2nd International Congress on Photosynthesis, Stresa, 1971, p. 507.
- 7) O. Koguchi and G. Tamura, *Agric. Biol. Chem.*, **52**, 373 (1988).
- 8) E. Saito, K. Wakasa, M. Okuma and G. Tamura, *Bulletin of the Association of Natural Science, Senshu University*, **3**, 45 (1970).
- 9) K. Tagawa and D. I. Arnon, *Nature*, **195**, 537 (1978).
- 10) M. Shin and R. Oshino, *J. Biochem.*, **83**, 357 (1978).
- 11) F. Yoneda, *Methods in Enzymology*, **66**, 267 (1980).
- 12) O. Koguchi, H. Takahashi and G. Tamura, *Agric. Biol. Chem.*, **52**, 1867 (1988).
- 13) U. K. Laemmli, *Nature*, **227**, 680 (1970).
- 14) L. Ornstein and B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, Art. 2, 305 (1964).
- 15) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
- 16) P. K. Nakane, *J. Histochem. Cytochem.*, **16**, 557 (1968).
- 17) L. M. Siegel and P. S. Davis, *J. Biol. Chem.*, **249**, 1587 (1974).
- 18) G. Tamura, K. Asada and R. S. Bandurski, *Plant Physiol.*, **42**, S36 (1967).
- 19) G. Tamura and S. Itoh, *Agric. Biol. Chem.*, **38**, 225 (1974).
- 20) G. Tamura, "Shokubutsu-Koso Tanpakushitu Kenkyuho." ed. by Y. Morita *et al.*, Kyoritsu Shuppan, Tokyo, 1976, p. 356.
- 21) J. Aketagawa and G. Tamura, *Agric. Biol. Chem.*, **21**, 2892 (1980).
- 22) R. J. Krueger and L. M. Siegel, *J. Biol. Chem.*, **21**, 2892 (1982).