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Cysteine synthase from a red alga *Porphyra yezoensis*: purification and properties

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Two types of cysteine synthase (CSase, EC 4.2.99.8) were purified from a red alga, *Porphyra yezoensis*. The enzymes (CSase-1 and 2) were separated by hydrophobic chromatography using Butyl-Toyopearl columns. The purified CSase-1 and 2 were judged homogeneous by PAGE and had specific activity of 408 and 300 μmol L-cysteine formed per min per mg protein, respectively. Both CSases had a molecular weight of 68,000 and consisted of two subunits with identical molecular weight of 34,000. The visible spectrum of the respective CSases shows the absorption maximum at 412 nm, indicating the presence of pyridoxal phosphate as a prosthetic group in the enzyme molecule. The same level of Km values (2.5 mM for *O*-acetyl-L-serine and 23 μM for sodium sulfide) was found in both of the *P. yezoensis* CSases. The optimum pH for the reaction was 8.0; however an apparent reduction of the CSase-2 activity in alkaline condition was observed. The antibody against purified CSase-1 was prepared for the immunochemical characterization of enzymes. The results of immunotitration and double immunodiffusion analyses suggested that the *P. yezoensis* CSases possesed antigenic determinants in common but some differences in their external construction.

Key Index Words: *cysteine synthase*—Porphyra yezoensis—*red algae*—*sulfur assimilation.*

Cysteine biosynthesis has been confirmed to proceed through sulfhydration of *O*-acetyl-L-serine (Kredich and Tomkins 1966, Yamagata 1976, Schmidt 1977). The enzymes catalyzing this reaction, cysteine synthases (CSases), have so far been studied mainly with a variety of higher plants (Masada *et al.* 1975, Tamura *et al.* 1976, Ascano and Nicholas 1977, Hock Ng and Anderson 1978, Murakoshi *et al.* 1985). Sulfide can act as the effective thiol donor for these CSases, and the reaction product has been shown to be L-cysteine in all cases.

Extensive purifications of the CSases have been accomplished with rape leaves (Masada *et al.* 1975, Nakamura and Tamura 1989) and radish roots (Tamura *et al.* 1976). Isolated CSase preparations reported so far exhibited similar absorption spectra with a peak in the vicinity of 410 nm, which was characteristic of pyridoxal phosphate containing proteins (Kredich and Tomkins 1966, Tamura *et al.* 1976, Leon *et al.* 1987).

Since little is known yet about the relevant enzymatic mechanism of the algal sulfide assimilation, the authors intended to purify CSase from a red alga, *Porphyra yezoensis*, which is one of the important edible seaweeds, and to characterize its enzymatic properties. In this report we describe the isolation and characterization of two hydrophobically distinguishable CSases from this alga.

Materials and Methods

Plant materials

Fresh thalli of *Porphyra yezoensis* Ueda, cultivated in Tokyo Bay in winter, were kindly supplied by Mr. S. Araki of the Yamamoto Nori Research Laboratory. The thalli were frozen and stored at −30°C until use.

Chemicals and supplies

DEAE-Toyopearl 650 M, Butyl-Toyopearl 650 S and Toyopearl HW-50 were supplied by TOSOH Co. Ltd. The following chemicals were products of commercial sources: Coomassie brilliant blue G-250 (Fluka AG); Calibration proteins kit (chymotrypsinogen A, ovalbumin and aldolase) (Boehringer Mannheim). Other chemicals were of analytical grade.

Enzyme assay

The assay method for cysteine synthase (CSase) activity was essentially the same as described in the previous paper (Nakamura and Tamura 1989). The reaction mixture contained, in a total volume of 1 m*l*, 50 μmol of potassium phosphate buffer, pH 8.0, 12.5 μmol of OAS, 5 μmol of sodium sulfide and an appropriate amount of enzyme. The reaction was started by the addition of enzyme and continued at 25°C for 10 min, then terminated by adding 1 m*l* of 4N HCl. L-Cysteine thus formed was measured by the method of Gaitonde (1967) using acid-ninhydrin reagent. One unit of CSase was defined as the amount of enzyme which produced 1 μmol of L-cysteine per min under the conditions described above.

Purification of enzyme

All the purification procedures were performed in a cold room maintained at 0-4°C. Dialysis was performed overnight against the indicated buffer and centrifugation was carried out at 10,000 *g* for 20 min.

Enzyme extraction from thalli

Fresh thalli of *P. yezoensis* were subjected to autolysis in twice the volume of buffer A (30 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA) 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. Two kilograms of fresh thalli was used in this purification experiment.

Ammonium sulfate fractionation

Solid ammonium sulfate was slowly added to the crude extract to 30% saturation. After standing for 60 min, the mixture was centrifuged and the inactive precipitate was discarded. Then, the supernatant was brought to 80% saturation with respect to ammonium sulfate, and the mixture was recentrifuged. The precipitate was collected and dissolved in a minimal amount of buffer A, and then dialyzed against the same buffer. By removing the insoluble material by centrifugation, a clear concentrated enzyme solution (260 m*l*) was obtained.

DEAE-Toyopearl column chromatography

The obtained enzyme solution was applied to a DEAE-Toyopearl column (5 cm diameter, 20 cm long) which had been equilibrated with buffer A. The elution was performed as follows. Linear concentration gradient of NaCl was established with 1 liter of buffer A in the mixing vessel and 1 liter of buffer A containing 200 mM NaCl in the reservoir. The active fractions were pooled.

First Butyl-Toyopearl 650S column chromatography

The pooled fraction (103 m*l*) was supplemented with ammonium sulfate to bring them to 30% of saturation. The precipitate formed was removed by centrifugation. The resulting supernatant solution was put on a Butyl-Toyopearl 650S column (2 cm diameter, 30 cm long), previously equilibrated with buffer A containing 30% saturated ammonium sulfate. After the column was washed with the same buffer, a linear concentration gradient of eluent was established with 300 m*l* of equilibrating buffer in the mixing vessel and the same volume of buffer A in the reservoir. The active fractions were combined and concentrated by ammonium sulfate precipitation (80% saturation). The precipitate, collected by centrifugation, was dissolved in a minimal amount of buffer A and then dialyzed against the same buffer.

Toyopearl HW-50 gel filtration

The dialyzed enzyme solution obtained

(2.8 m*l*) was filtered through a Toyopearl HW-50 column (2 cm diameter, 90 cm long), equilibrated with buffer A, and the active fractions were pooled.

Second Butyl-Toyopearl 650S column chromatography

The combined enzyme solution (10.7 m*l*) was brought to 25% saturation with ammonium sulfate and applied to a Butyl-Toyopearl 650S column (1.3 cm diameter, 15 cm long), previously equilibrated with buffer A, containing 25% saturated ammonium sulfate. A linear gradient of ammonium sulfate concentration was established with 200 m*l* of 25% saturated ammonium sulfate solution, containing enough buffer A to bring the solution to pH 8.0 in the mixing vessel and 200 ml of 10% saturated ammonium sulfate solution containing the same buffer in the reservoir.

Third Butyl-Toyopearl 650S column chromatography

The pooled fraction (62 m*l*) was supplemented with ammonium sulfate to bring them to 25% saturation. The resulted solution was applied to a Butyl-Toyopearl 650S column (1 cm diameter, 23 cm long), previously equilibrated with buffer A containing 25% saturated ammonium sulfate. The chromatographic procedures were essentially the same as described for the second Butyl-Toyopearl 650S column chromatography with a slight modification; the gradient slope of ammonium sulfate was 25-15% instead of 25-10%.

Determination of molecular weight

The molecular weight of the enzyme was estimated by HPLC CCPM system (TOSOH Co. Ltd.) using TSK gel G3000SW column (0.75 cm diameter, 30 cm long), previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 200 mM sodium sulfate.

Preparation of antiserum and immunochemical characterization

Homogeneous CSase-1 from *P. yezoensis* thalli (ca. 400 μg) emulsified with complete Freund's adjuvant (Difco Laboratories) was administrated subcutaneously to a New Zealand white rabbit. The booster injections were performed four times at two week intervals with the same amount of antigen. Two weeks after the final injection, whole blood was collected and allowed to clot at 25°C for 1 hr and then at 4°C overnight. Serum was separated by centrifugation. The antiserum obtained above was used for the experiments without further purification. Immunotitration was performed by incubating antiserum with enzyme sample for 10 min at 25°C. Then, CSase activity was assayed according to the standard assay system. The double immunodiffusion was performed by the method of Ouchterlony and Nilson (1973).

Other analytical methods

Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard. Analytical gel electrophoresis was carried out according to the method of Davis (1964) for PAGE and Laemmli (1970) for SDS-PAGE. Protein bands on the gel slab were stained by Coomassie brillinat blue R-250.

Results and Discussion

Most information on the sulfate assimilation by higher plants has been derived from experiments using leaf and root tissues, and sulfhydration of *O*-acetyl-L-serine, catalyzed by cysteine synthase, is generally considered to be the final step of cysteine biosynthesis in plant tissues. However, such data from algae are very scanty.

Enzyme purification

Table 1 shows a summary of the purification starting from 2 kg of fresh *P. yezoensis* thalli. The CSase with the lower hydrophobicity, obtained from the third Butyl-Toyopearl chromatography step (Fig. 1), represents about 3/4 of the total CSase and will be identified in further characterization

Table 1. Summary of purification of cysteine synthase from a red alga, *Porphyra yezoensis*.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Crude extract	3,358	9,138	0.37	100	1
Ammonium sulfate	4,186	6,266	0.67	125	2
DEAE-Toyopearl	3,011	413	7.29	90	20
1st Butyl-Toyopearl	1,926	14.7	131	57	354
Toyopearl HW-50	1,199	5.6	214	36	578
2nd Butyl-Toyopearl	801	2.5	320	24	865
3rd Butyl-Toyopearl					
CSase-1	265	0.65	408	8	1,103
CSase-2	81	0.27	300	2	810

and discussion with suffix 1. The other enzyme will have suffix 2. The procedure, described here, allows 1,103 and 810-fold purification of *P. yezoensis* CSases (CSase-1 and 2) with recovery of 8 and 2%, respectively. Both isoforms showed specific activities of 408 and 300 μmol L-cysteine formed per min per mg protein. These values were much higher than those of purified algal cysteine synthase from other sources: from a blue-green alga *Synechococcus* sp. (Diessener and Schmidt 1981) and a green alga *Chlamydomonas reinhardtii* (Leon *et al.* 1987), approx. 1 and 41.2 μmol L-cysteine formed per min per mg protein, respectively. When the purified enzymes were separately subjected to native PAGE, a single intensely stained protein band was observed (Fig. 2a).

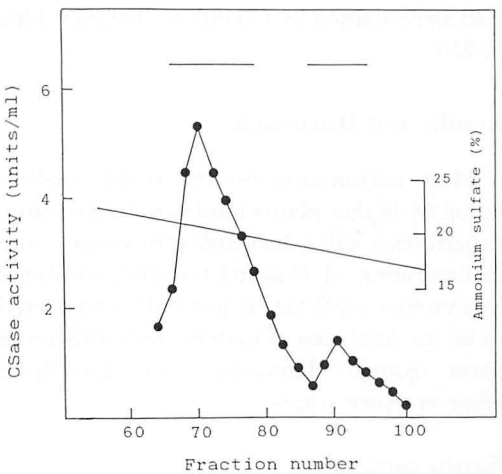

Fig. 1. Elution pattern of CSase from the 3rd Butyl-Toyopearl column. Experimental conditions were essentially the same as that of the 1st Butyl-Toyopearl chromatography, except for the gradient concentration of 25–15% of ammonium sulfate. Fractions of 2.7 m*l* were collected and the tubes in brackets, Nos. 66–78 and 88–96, were pooled as CSase-1 and CSase-2 purified preparations, respectively. —●—, CSase activity.

Molecular weight

The molecular weights of the purified CSase-1 and 2 were estimated by HPLC using TSK gel column G3000SW. In Fig. 3, the elution volume for the marker proteins are plotted against the molecular weights. The elution volume for these CSase isoforms was equally 10.6 m*l*, corresponding to a molecular weight of 68,000. The molecular weights of highly purified plant CSases so far reported

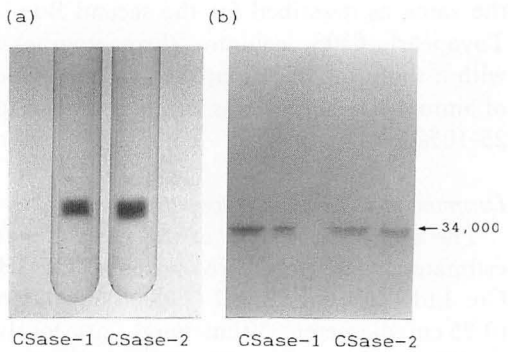

Fig. 2. Electrophoretogram of purified CSases on PAGE (a) and SDS-PAGE (b). Purified CSase preparations (30 μg for PAGE, 6 and 3 μg for SDS-PAGE) were subjected to electrophoresis on 7% poly-acrylamide gel disc (a) or 15% SDS-polyacrylamide gel slab (b). Protein bands were stained with Coomassie brilliant blue R-250.

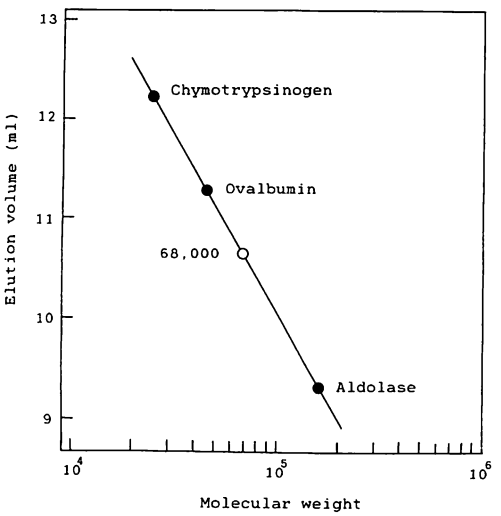

Fig. 3. Determination of the molecular weight of CSases by HPLC using TSK Gel G3000SW. The elution was performed with 50 mM potassium phosphate buffer, pH 7.0, containing 200 mM sodium sulfate with a flow rate of 0.5 m*l* per min. The detection of the enzymes and marker proteins was carried out by monitoring the absorbance at 280 nm.

are in the range of 56,000-70,000: by Masada *et al.* (1975) for rape leaves, by Tamura *et al.* (1976) for radish roots and several other plant CSases reported previously.

The subunit molecular weights of these enzymes were estimated by SDS-PAGE. Each preparation gave a single protein band on SDS-PAGE gel (Fig. 2b). The *Rf* values for both CSase-1 and 2 were equally 0.45, corresponding to a molecular weight of 34,000. So, it might be concluded that the purified hydrophobically distinct *P. yezoensis* CSases appear to be composed of two subunits with the same molecular weight.

Absorption spectrum

The solution of CSase-1 and 2 separately gave an absorption spectrum as shown in Fig. 4. In addition to the protein peak at 280 nm, the purified CSases exhibited absorption maximum at 412 nm. The ratios of the absorbance at 412 to 280 nm are 0.22 for CSase-1 and 0.13 for CSase-2, respectively. Since this absorption spectrum is similar to that reported by Masada *et al.* (1975) for rape leaf CSase (pyridoxal phosphate, PLP, containing enzyme), it can be concluded that respective two CSases from *P. yezoensis* equally have PLP as a prosthetic group in the molecule. This fact suggests that CSases from higher plants and algal sources may have basically similar functional groups.

Effect of pH and buffer composition on the CSase activity

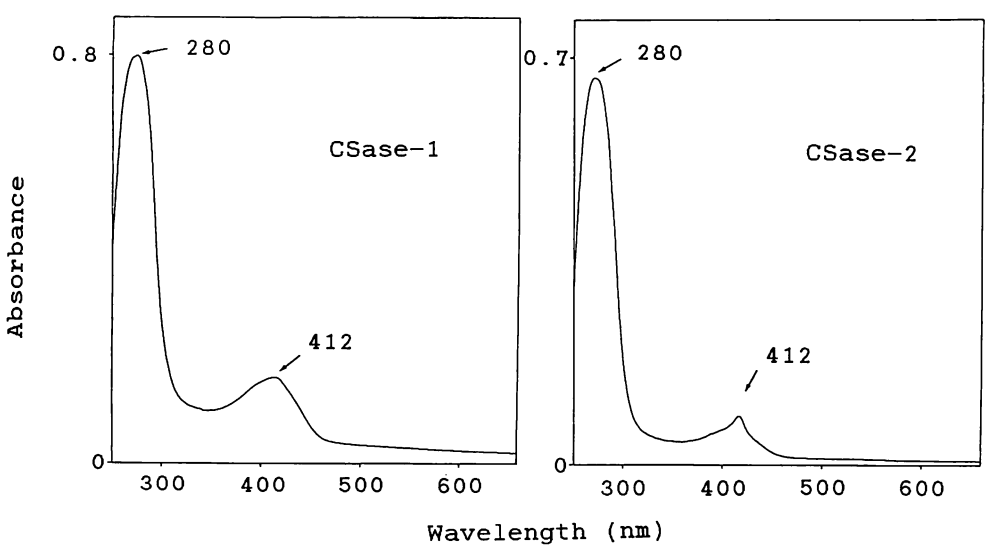

Fig. 4. Absorption spectra of CSases. The purified preparations were dialyzed against buffer A containing 100 mM NaCl. The spectra were measured at room temperature.

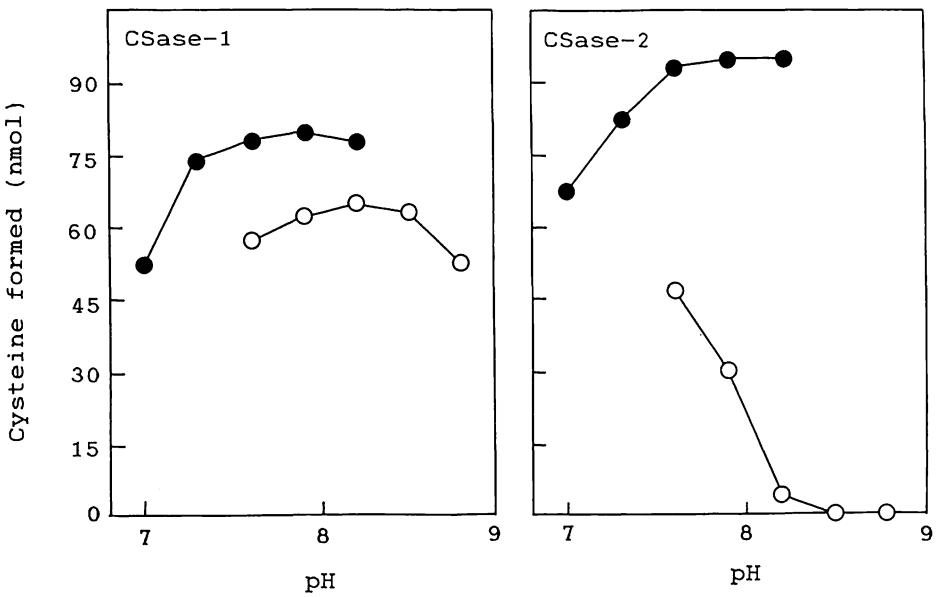

Fig. 5. Effects of pH on CSase activity. The assay procedures were described in the text. Tris-HCl buffer (-○-) and potassium phosphate buffer (-●-) were used in the assay.

The effect of pH on the CSase activity was studied under the same conditions as described in the Materials and Methods section, except that pH was varied as indicated (enzyme concentration, 0.2 μg for CSase-1 and 0.3 μg for CSase-2 per reaction mixture). As shown in Fig. 5, in which 100 mM potassium phosphate buffer was used, the pH-activity curves obtained for CSase-1 and 2 were rather broad and showed an optimum pH around 8.0. And a similar observation has been reported for the enzyme from a number of higher plants (Masada *et al.* 1975, Hock Ng and Anderson 1978, Murakoshi *et al.* 1985). On the other hand, in Tris-HCl buffer, the enzymatic activity of CSase-2 at pH 8.0 was about 0.35 times as low as that observable in phosphate buffer at the same pH, and this CSase-2 activity in Tris-HCl buffer became much decreased in alkaline region. This effect of pH for CSase-2 reaction has not been reported in plant enzymes; however, it was analogous to the result of a bispecific enzyme in yeast (Yamagata *et al.* 1974) which functioned as *O*-acetyl-L-serine (OAS) and *O*-acetyl-L-homoserine sulfhydrylase.

Heat stability

As for the heat stability of *P. yezoensis* CSases, they exhibited similar behavior: they were stable on the heat treatment at 45°C for 10 min, but 90-95% of the activities were decreased at 60°C for 10 min (data not shown). It showed that *P. yezoensis* CSases were less stable on the heat treatment than other plant CSases reported previously. For instance, CSase from rape leaves (Masada *et al.* 1975) and radish roots (Tamura *et al.* 1976) were stable at 70°C for 3 min and 65°C for 2 min, respectively.

Effect of substrate concentration

The effects of substrates concentration for the isoforms were also determined. From the double reciprocal plots for OAS and sodium sulfide, respective CSases showed the same range of Km values, 2.5 mM for OAS and 23 μM for sodium sulfide.

Immunological comparison of CSases

Behavior of two CSases towards the anti-CSase-1 was studied by the inhibition of enzymatic activity and double immunodiffusion. Fig. 6 shows the immunotitration

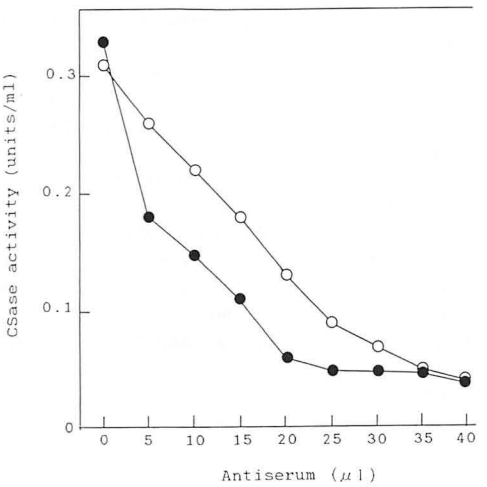

Fig. 6. Immunotitration curves of CSases using anti-CSase-1 serum. Each of 0.33 units/m*l* of CSase-1 and 0.31 units/m*l* of CSase-2 was used for the experiment. —●—, CSase-1; —○—, CSase-2.

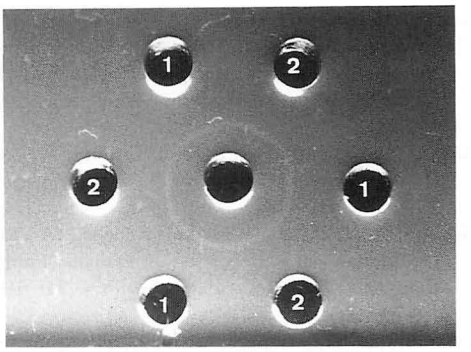

Fig. 7. Ouchterlony double immunodiffusion analysis of CSase-1 and 2. Six μl wells were cut in the agar plate (1.2%, dissolved in 20 mM potassium phosphate buffer, pH 7.2, containing 150 mM NaCl and 0.05% sodium azide) filled with purified CSases as shown: well 1, CSase-1; well 2, CSase-2. Each preparations contain CSase activity around 10 units/m*l*. The center well contained 6 μl of anti-CSase-1 serum.

curves of CSase-1 and 2. With the enzyme samples, activity in the incubated solution decreased with increasing amounts of the antiserum. These activities were found to be differentially inhibited by the antiserum. CSase-1 activity was more susceptible to inhibition by antiserum than CSase-2 activity. The amount of the antiserum which caused 50% inhibition of CSase activity was 7.5 μl for CSase-1 and 17.5 μl for CSase-2. The comparison of antigenisity between CSase-1 and 2 was analyzed by Ouchterlony double immunodiffusion. As shown in Fig. 7, using anti-CSase-1 serum, a single precipitin band which was completely fused was found. The results of these two immunochemical experiments suggest that *P. yesoensis* CSases have the same antigenic determinants in common, but some difference in their external constructions may present.

It remains to be established if the difference between *P. yezoensis* CSase-1 and 2 is due to the different primary structure, i.e. that they are isozymes, or if CSase-1 or 2 is derived from another one in early step of purification. Dissener and Schmidit (1981) have also observed the occurrence of a marked twin bands in the electrophoretic pattern of partially purified CSase from *Synechococcus*. These authors did not identify those twin bands as the two isozymes, but the presence of two CSases might be a common phenomenon in the behavior of algal CSase.

References

Ascano, A. and Nicholas, D. J. D. 1977. Purification and properties of *O*-acetyl-L-serine sulfhydrylase from wheat leaves. Phytochemistry **16:** 889–893.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

Davis, B. J. 1964. Disk electrophoresis. II. Methods and application to human serum proteins. Ann. N. Y. Acad. Sci. **121:** 404–427.

Diessener, W. and Schmidt, A. 1981. Isoenzymes of cysteine synthase in the cyanobacterium *Synechococcus* 6301. Z. Pflanzenphysiol. **102:** 57–68.

Gaitonde, M. K. 1967. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally amino acids. Biochem. J. **104:** 627–633.

Hock Ng, B. and Anderson, J. W. 1978. Chloroplast cysteine synthases of *Trifolium repens* and *Pisum sativum*. Phytochemistry **17:** 879–885.

Kredich, N. M. and Tomkins, G. M. 1966. The enzymic synthesis of L-cysteine in *Escherichia coli* and *Salmonella typhimurium*. J. Biol. Chem. **241:** 4955–4965.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

Leon, J., Romero, F., Galvan, F. and Vega, J. M. 1987. Purification and physicochemical characterization of *O*-acetyl-L-serine sulfhydrylase from *Chlamydomonas reinhardtii*. Plant Science **53**: 93–99.

Masada, M., Fukushima, K. and Tamura, G. 1975. Cysteine synthase from rape leaves. J. Biochem. **77**: 1107–1115.

Murakoshi, I., Ikegami, F. and Kaneko, M. 1985. Purification and properties of cysteine synthase from *Spinacia oleracea*. Phytochemistry **24**: 1907–1911.

Nakamura, K. and Tamura, G. 1989. Five isoforms of cysteine synthase in rape leaves. Agrc. Biol. Chem. **53**: 2537–2538.

Ouchterlony, O. and Nilsson, L. A. 1973. Immunodiffusion and immunoelectrophoresis. *In* D.M. Meir (Ed.) Handbook of Experimental Immunology, 3rd Ed. Blackwell, Oxford.

Schmidt, A. 1977. Exchange of cysteine-bound sulfide with free sulfide and cysteine synthase activity in *Chlorella pyrenoidosa*. Z. Pflanzenphysiol. **84**: 435–449.

Tamura, G., Iwasawa, T., Masada, M. and Fukushima, K. 1976. Some properties of cysteine synthase from radish roots. Agric. Biol. Chem. **40**: 637–638.

Yamagata, S. 1976. *O*-acetylserine and *O*-Acetylhomoserine sulfhydrylase of yeast subunit structure. J. Biochem. **80**: 787–797.

Yamagata, S., Takeshima, K. and Naiki, N. 1974. Evidence for the identity of *O*-acetylserine sulfhydrylase with *O*-acetyl-homoserine sulfhydrylase in yeast. J. Biochem. **75**: 1221–1229.

藤森　泰*・中村勝人・田村五郎*：紅藻スサビノリのシステイン合成酵素の精製とその性質**

古来から食品として利用されている紅藻 *Porphyra* は，タンパク質に富み，また，含硫アミノ酸を豊富に含む食品である。含硫アミノ酸システインの合成酵素 (CSase) については，高等植物では詳細な研究がなされているが，藻類を対象とした研究は少ない。そこで，スサビノリ (*Porphyra yezoensis*) の粗抽出液を硫安分画，DEAE-Toyopearl カラムクロマト，ゲルろ過，Butyl-Toyopearl カラムクロマトで精製することにより電気泳動的に均一な2種の CSase (1, 2) を確認した。比活性はそれぞれ408及び300，分子量は共に68,000であり，SDS-PAGE より分子量34,000の同一サブユニットから成る二量体であることが示された。反応の至適 pH は CSase-1, 2 共に8.0，Tris-HCl 緩衝液においてはアルカリ側で明らかな活性の差が認められた。また，抗 CSase-1 血清を作製し免疫学的比較も試みた。(*271 千葉県松戸市松戸648　千葉大学園芸学部生物化学研究室，**505-11 岐阜県岐阜市柳戸1-1　岐阜大学教養部生物学教室)