Purification and Characterization of a Novel Cysteine Synthase Isozyme from Spinach Hydrated Seeds

Takayuki YAMAGUCHI,^{1,2} Xia ZHU,¹ and Masahiro MASADA^{1,*}

¹Department of Bioresources Chemistry, Faculty of Horticulture, Chiba University, Matsudo 648, Chiba 271, Japan

²Engineering Department, Kazami Co., Ltd., Sukedo 1-26, Tochigi 326, Japan

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A novel type of cysteine synthase (CSase, EC 4.2.99.8) isozyme, designated as CSase 1', was purified to homogeneity from hydrated spinach seeds. The enzyme had a molecular weight of 68,000 and consisted of two identical subunits of M_r 34,000. The apparent K_m for *O*-acetyl-L-serine was 8.33 mM and that for sulfide was 0.66 mM. The activity of CSase 1' was maintained when it was treated at 60°C for 1 min. This novel enzyme was similar to CSases 1, 2, and 3 already purified from spinach leaves, in results of double immunodiffusion, molecular weight, subunit composition, K_m values for *O*-acetyl-L-serine and sulfide, and heat stability. On the other hand, *N*-terminal amino acid sequence, effects of immunotitration, pH optimum, and effects of hydroxylamine on purified CSase 1' were different from those of the other CSases. Furthermore, it was found that CSases 2S and 3S isolated from hydrated spinach seeds were identical with the CSases 2 and 3 reported previously. It was also disclosed that CSases 1, 2, and 3 were localized in chloroplasts, cytosol, and mitochondria, respectively.

Key words: cysteine biosynthesis; cysteine synthase; *O*-acetylserine(thiol)lyase; *O*-acetyl-L-serine; sulfate assimilation

In higher plants and microorganisms, reduced inorganic sulfate is assimilated to cysteine by the sulfate assimilation pathway, and cysteine is the precursor of many sulfur-containing organic compounds necessary for growth and metabolism.¹⁾ The incorporation of a reduced inorganic sulfur involves two enzymatic reactions.^{2–4)} The first step, the formation of *O*-acetyl-L-serine (OAS) from serine and acetyl-CoA, is catalyzed by serine acetyltransferase (SATase; EC 2.3.1.30). Cysteine synthase (CSase; EC 4.2.99.8) catalyzes the second reaction which is the formation of L-cysteine from OAS and sulfide.

With respect to the study for CSase, it is well known that plural CSases are present among different species.^{5–10)} These CSases have been individually purified from several species and respective property has been also reported.^{5–15)} Concerned with CSase in spinach leaves, it was reported that CSase activity was demon-

strated in chloroplasts, mitochondria, and cytosol.¹⁶⁾ Recently, the genes for CSase isozymes A, B, and C localized in cytosol, chloroplasts, and mitochondria, respectively, have been cloned from spinach.^{17–20)}

In the previous work we purified three CSase isozymes from spinach leaves, and designated them CSase 1, CSase 2, and CSase 3 with reference to the mobility on native PAGE.²¹⁾ Each isozyme has a molecular mass of 63,000 and consists of two identical subunits of M_r 34,000 for CSase 1 and CSase 3 and 35,000 for CSase 2. These isozymes were revealed to have different primary structures by both amino acid analysis and results of digestion with proteinase. In addition, we found that CSase 1 was localized in chloroplasts and not detected in hydrated seeds and that CSase 1 was identical with CSase B. CSases 2 and 3 were not identified with CSases A and C.^{21,22)} During seed germination, a novel CSase designated CSase 1' was found; it was different from CSases 1, 2, and 3 in terms of electrophoretic mobility on native PAGE.²²⁾ CSase 1' was detected only during the initial period of germination and disappeared after 6 or 7 days of development. Interestingly, the timing of the expression of CSase 1' differs from those of CSases 1, 2, and 3.

To investigate the characteristics and physiological functions of CSase 1', we have first purified CSase 1' from hydrated spinach seeds and sequenced the *N*-terminal amino acids of CSase 1' and other CSase isozymes (CSases 1–3) from leaves. Here, we report the purification and characterization of CSase 1', and compare its properties with those of CSases 1, 2, and 3. We also demonstrate the localization of CSases 1, 2, and 3.

Materials and Methods

Materials. Fresh spinach leaves and seeds were obtained from a local market. CSases 1, 2, and 3 were purified to homogeneity from spinach leaves according to the method described in the previous paper.²¹⁾ *O*-Acetyl-L-serine was prepared by the method of Nagai and Flavin.²³⁾ Polyclonal antibody against CSases 1, 2, and 3 was prepared in rabbits according to the previous method.²²⁾ Other chemicals were obtained from commercial sources.

* To whom correspondence should be addressed: Faculty of Horticulture, Chiba University, Matsudo 648, Chiba 271, Japan. Fax. 81-47-363-1497.

Abbreviations: CSase, cysteine synthase; SATase, serine acetyltransferase; OAS, *O*-acetyl-L-serine; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; 2-ME, 2-mercaptoethanol; PBS, 20 mM potassium phosphate buffer (pH 7.2) containing 150 mM NaCl.

Assay for cysteine synthase activity. The enzyme activity was measured in a volume of 1 ml containing 50 mM potassium phosphate buffer (pH 8.0), 12.5 mM OAS, 3 mM sodium sulfide, and an appropriate amount of enzyme. Following preincubation for 2 min at 25°C, the reaction was initiated by the addition of enzyme solution, continued for 10 min at 25°C, and stopped by the addition of 1 ml of 4 N HCl. L-Cysteine formed was determined by the method of Gaitonde.²⁴⁾ One unit of CSase activity was defined as the amount of enzyme which produced 1 μ mol of L-cysteine per min.

Preparation of crude extract. All procedures were carried out at 4°C. Unless otherwise indicated, all of the buffer solutions contained 0.5 mM EDTA and 10 mM 2-ME. After dry seeds (550 g) were soaked overnight in distilled water, the hydrated seeds (1 kg) were homogenized with two volumes of 200 mM potassium phosphate buffer (pH 8.0) containing 1 mM PMSF and 5% (w/v) Polyclar AT in an Ace homogenizer (Nihon Seiki Co., Tokyo, Japan). The homogenate was squeezed through two layers of cheesecloth with the aid of a hand press, and the crude extract was obtained.

Heat treatment. After the crude extract was brought to 60°C in a large water bath at 100°C with stirring, the solution was transferred to a 60°C incubator and held at 60°C for 1 min. After immediate chilling, the solution was centrifuged at 10,000 $\times g$ for 20 min. The supernatant obtained was collected.

Ammonium sulfate fractionation. Crystalline ammonium sulfate was added slowly to the above supernatant with stirring until 35% saturation was achieved. The mixture was allowed to stand for 60 min and then centrifuged at 10,000 $\times g$ for 20 min. Additional ammonium sulfate was added to the supernatant obtained with stirring until 80% saturation. After being left to stand for 60 min, the resulting precipitate was collected by centrifugation at 10,000 $\times g$ for 20 min. The precipitate was dissolved in a small volume of 30 mM Tris-HCl buffer (pH 8.0), and then dialyzed overnight against the same buffer.

DEAE-Toyopearl column chromatography. The dialyzed enzyme solution was applied to a DEAE-Toyopearl column (2.8 $\times$ 34 cm), previously equilibrated with the dialyzing buffer. After the column was thoroughly washed with the same buffer, proteins were eluted by means of a linear gradient of increasing concentration of NaCl from 0 to 300 mM. The total amount of eluting fluid was 800 ml, and the flow rate was maintained at 0.6 ml per min (10 ml fractions). The active fractions were pooled.

Butyl-Toyopearl column chromatography. The solution obtained was supplemented with ammonium sulfate to 30% saturation. The precipitate formed was removed by centrifugation. The supernatant obtained was applied to a hydrophobic column of Butyl-Toyopearl 650S (3.3 $\times$ 23.5 cm) equilibrated with 30 mM Tris-HCl

buffer (pH 8.0) containing 30% saturated ammonium sulfate. After the column was washed with the same buffer, proteins were eluted by an ammonium sulfate gradient from 30% to 0% saturation. The total amount of eluting fluid was 900 ml, and the flow rate was maintained at 0.6 ml per min (7 ml fractions). The active fractions were pooled, and the proteins were precipitated by saturation with ammonium sulfate. The precipitate collected by centrifugation was dissolved in a small volume of 30 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl and dialyzed against the same buffer.

Ultrogel AcA44 gel filtration. The dialyzed enzyme solution was applied to a column (2.2 $\times$ 81.7 cm) of Ultrogel AcA44 equilibrated with the same buffer as above. Elution was performed with the equilibrating buffer at a flow rate of 0.28 ml per min with a fraction size of 3.5 ml. Fractions with high specific activity were combined.

Blue-Sepharose column chromatography. The solution obtained was diluted to five volumes with 30 mM Tris-HCl buffer (pH 8.0), and the diluent was applied to a column (1.6 $\times$ 10 cm) of Blue-Sepharose equilibrated with the same buffer. After the column was washed with the same buffer, proteins were eluted with a linear concentration gradient of the eluant established with 250 ml of 30 mM Tris-HCl buffer (pH 8.0) in a mixing vessel and 250 ml of the same buffer containing 2 M NaCl in a reservoir. The flow rate was maintained at 0.6 ml per min (2.4 ml fractions). The active fractions were pooled as the Blue-Sepharose fraction.

Superdex 200 column chromatography. The solution obtained was concentrated by dialysis against polyethylene glycol 20,000. The concentrated enzyme solution was then dialyzed against 30 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. The supernatant obtained by centrifugation was applied to a Superdex 200 column (1.6 $\times$ 60 cm, Pharmacia) equilibrated with the same buffer. Elution was performed with the equilibrating buffer at a flow rate of 0.8 ml per min with a fraction size of 1 ml. Fractions with high specific activity were combined.

Mono Q column chromatography. The solution obtained was concentrated by ultrafiltration and dialyzed against 30 mM Tris-HCl buffer (pH 8.0). The dialyzate was applied to a Mono Q column (0.5 $\times$ 5 cm), equilibrated with the same buffer. After the column was washed with the same buffer, proteins were eluted by means of a linear gradient of increasing concentration of NaCl from 0 to 300 mM. The total amount of eluting fluid was 30 ml, and the flow rate was maintained at 1.0 ml per min (0.5 ml fractions). The active fractions were pooled.

Preparative PAGE. Preparative PAGE was performed by the system of disc preparative electrophoresis (NA-1800, Nihon Eido Co., Tokyo, Japan), using 7.5% polyacrylamide gel. The solution obtained was concen-

trated and subjected to the PAGE. The eluate corresponding to each CSase isozyme was pooled and used for subsequent analyses as the purified enzyme preparation.

Amino acid analysis. The purified enzymes were hydrolyzed in a sealed tube in 0.3 ml of 6 M HCl at 110°C for 24 h using an Amino Acid Hydrolysis Station AHST-1 (Shimadzu). Amino acid analysis was carried out on a high-performance amino acid analyzer (ATTO MLC-703).

Amino acid sequence analysis. Each CSase isozyme purified from spinach leaves and hydrated seeds was blotted on a PVDF membrane for protein sequencing. The sequence of amino acids at the *N*-terminus was identified on a Shimadzu PPS-10 protein sequencer.

Immunological test. Double immunodiffusion analysis was done by the method of Ouchterlony.²⁵⁾ Immunotitration was done by incubating antiserum or control serum (150 μ l) with an enzyme sample (200 μ l) for 15 h at 4°C. The mixture was centrifuged at 10,000 $\times g$ for 10 min and then CSase activity in the supernatant was determined according to the standard assay system.

Other analytical methods. Protein was measured by the method of Bradford²⁶⁾ using the protein assay reagent with bovine serum albumin as a standard. Analytical gel electrophoresis was carried out by the method of Davis²⁷⁾ for native PAGE and by that of Laemmli²⁸⁾ for SDS-PAGE. Protein bands on the gel slab were stained with Coomassie Brilliant Blue R-250.

Results

Summary of purification

Three CSase isozymes were isolated by preparative PAGE from hydrated seeds. These CSases were designated as CSases 1', 2S, and 3S with reference to the mobility on native PAGE. Table I shows a summary of a typical experiment. The specific activities were 834 units/mg protein for CSase 1', 1000 units/mg protein for CSase 2S, and 1820 units/mg protein for CSase 3S. Each

Fig. 1. Electrophoretograms of CSase on PAGE and SDS-PAGE.

(A) About 3 μ g of each protein was subjected to electrophoresis at 4°C on 7.5% polyacrylamide gel was done. A constant current of 10 mA was applied for 4 h. (B) About 3 μ g of CSase 1' was incubated at 100°C in a water bath for 3 min with 0.1% SDS and 1% 2-ME. The solution was subjected to electrophoresis on a 15% SDS-polyacrylamide gel. A constant current of 10 mA was applied for 4 h. Protein bands were stained with Coomassie brilliant blue R-250. lane 1, CSase 1; lane 1', CSase 1'; lane 2, CSase 2S; lane 3, CSase 3S; lane M, Marker proteins.

purified enzyme preparation was recognized to be homogeneous by native PAGE (Fig. 1A).

Properties of purified isozyme

Molecular weight

The subunit molecular weight was estimated by SDS-PAGE with 15% polyacrylamide gel. As shown in Fig. 1B, the subunit molecular weight was calculated to be about 34,000. The molecular weight of CSase 1' was estimated by gel filtration through a Superdex 200 column (1.6 $\times$ 60 cm, Pharmacia) equilibrated with 30 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. The molecular weight of CSase 1' was calculated to be about 68,000 (Fig. 2). From these results it appears that CSase 1' was composed of two identical subunits as in the case of other CSase isozymes. The molecular weights of CSases 2S and 3S were calculated to be about 63,000 in a similar manner as above (data not shown). The subunit molecular weights and subunit structures of CSases 2S and 3S from hydrated seeds were determined to be the

Table I. Purification of Cysteine Synthase from Hydrated Spinach Seeds

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)
Crude extract*	2030	7360	0.28	100
Heat treatment	1460	5050	0.29	71.9
Ammonium sulfate	1090	924	1.18	53.7
DEAE-Toyopearl	1000	177	5.65	49.3
Butyl-Toyopearl	847	45.6	18.6	41.7
Ultrogel AcA 44	558	14.2	39.3	27.5
Blue-Sepharose	376	4.80	78.3	18.5
Superdex 200 pg	84.0	0.974	86.2	4.1
Mono Q	27.6	0.0142	1940	1.4
Preparative PAGE				
CSase 1'	2.92	0.0035	834	0.14
CSase 2S	5.42	0.0054	1000	0.27
CSase 3S	8.36	0.0046	1820	0.41

* From 1.0 kg of hydrated spinach seeds (dry weight, 550 g).

Fig. 2. Determination of Molecular Weight of CSase 1'.

CSase (45 $\mu\text{g/ml}$) and marker proteins (10 mg/ml) were separately filtered through a Superdex 200 column (1.6 $\times$ 60 cm) equilibrated with 30 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. a, BSA dimer (136,000); b, BSA monomer (68,000); c, ovalbumin (45,000); d, chymotrypsinogen (25,000); e, cytochrome c (12,500).

same as those of CSases 2 and 3 from leaves²¹⁾ (data not shown).

Effects of substrate concentration on enzyme activity

The effects of OAS and sulfide on CSase 1' were determined by varying their concentrations, 0–20 mM for OAS and 0–1.6 mM for sulfide. Michaelis-Menten kinetics behavior was observed (data not shown). The K_m values for CSase 1' were calculated to be about 8.33 mM with OAS and 0.66 mM with sulfide, respectively, which were determined from the Lineweaver-Burk plots of the data. Moreover, The K_m values for CSases 2S and 3S were examined, which were 5.56 and 5.26 mM with OAS, 1.25 and 2.50 mM with sulfide, respectively. These values for CSases 2S and 3S from hydrated seeds were the same as those of CSases 2 and 3 purified from spinach leaves.²¹⁾

pH optimum

Effects of both pH and buffer compositions on the CSase 1' activity were studied using phosphate and Tris-

HCl buffers, and results were compared with those for other CSase isozymes. CSases 2S and 3S used were purified from hydrated seeds, and CSase 1 was from spinach leaves. As shown in Fig. 3, the pattern of the pH-rate curve for CSase 1' was different from that of other CSase isozymes which showed similar profiles. The patterns of the pH-rate curves for CSases 2S and 3S from hydrated seeds were similar to those of CSases 2 and 3 from spinach leaves (data not shown).

Heat stability

The purified enzyme, CSase 1', was treated at 60°C for 1 min. The enzyme activity was maintained as in the case as CSases 1, 2S, and 3S. CSase 1' was stable in the treatment at 60°C for 2 min, because 90% of CSase 1' activity remained. Under the same conditions, 43%, 70%, and 90% of activity were remained for CSase 1, 2S, and 3S, respectively. However, after treatment at 70°C for 2 min, even though about 10% of CSase 1' activity remained, but the activity of CSases 1, 2, and 3 was almost lost. The heat stability of CSases 2S and 3S from hydrated seeds was almost the same as those of CSases 2 and 3 from leaves (data not shown).

Effects of hydroxylamine concentration on enzyme activity

CSase activity was inhibited by hydroxylamine which is a potent inhibitor of pyridoxal phosphate enzyme. As shown in Fig. 4, the activities of CSases 2S and 3S were almost completely inhibited, but 13% of CSase 1 activity remained at a concentration of 10 mM. However, hydroxylamine was not so effective for CSase 1' as compared with the cases of other CSase isozymes. With 10 mM hydroxylamine, about 43% of CSase 1' activity remained. The inhibitory patterns for CSases 2S and 3S purified from hydrated seeds were similar to those of CSases 2 and 3 purified from spinach leaves, respectively.

Immunological test

The precipitin lines in Ouchterlony double im-

Fig. 3. Dependence of CSase Reaction Rate on pH.

The assay procedures are described in the text. Potassium phosphate buffer (A) and Tris-HCl buffer (B) were each used at a final concentration of 0.1 M. ●, CSase 1; ▲, CSase 2S; ■, CSase 3S; ○, CSase 1'.

Fig. 4. Effects of Hydroxylamine Concentration on Enzyme Activity.

Purified enzymes (0.2–2 μ g) was used in each reaction. In the control, hydroxylamine was omitted; the activity is taken to be 100%. Other experimental conditions are described in the text. ●, CSase 1; ▲, CSase 2S; ■, CSase 3S; ○, CSase 1'.

munodiffusion were completely fused, indicating that the antigens (CSase 1', 2S, and 3S from hydrated seeds and CSase 1 from green leaves) had identical epitopes (Fig. 5). To investigate the relation between CSase 1' and the other CSases (CSases 2S and 3S from hydrated seeds and CSase 1 from green leaves), the immunotitration of each isozyme with a polyclonal antibody was also carried out. As shown in Fig. 6, the decreasing pattern of CSase 1' activity was not in agreement with that of the other isozymes.

Amino acid composition

The amino acid compositions of the purified CSases are summarized in Table II. Since the amino acid analysis was carried out on an acid-hydrolyzed sample, tryptophan and cysteine could not be determined. The most apparent differences between CSase 1' and other three CSases were in the amounts of Thr, Ser, Pro, Gly, Met, Lys, His, and Arg. The amino acid compositions of CSases 2S and 3S purified hydrated seeds were consistent with those of CSases 2 and 3 from leaves (data not shown).

Fig. 5. Ouchterlony Double Immunodiffusion Analysis of CSase Isozymes.

Five-microliter wells were cut in the agar plate (1.2%, dissolved in PBS containing 0.05% sodium azide) and filled with purified CSases: Well 1, CSase 1; well 1', CSase 1'; well 2, CSase 2S; well 3, CSase 3S. Each preparation contained about 5 units/ml of CSase activity. The center well contained 5 μ l of anti-CSase antiserum.

Fig. 6. Immunotitration Curves of CSase Isozymes Using Anti-CSase Antiserum.

Each preparation of CSase isozymes contained about 200 μ l of 0.45 units/ml of CSase activity. ●, CSase 1; ▲, CSase 2S; ■, CSase 3S; ○, CSase 1'.

Amino acid sequence analysis

The *N*-terminal sequences of the four CSase isozymes (CSases 1, 1', 2, and 3) are shown in Table III. These amino acid sequences were different from each other. The *N*-terminal sequences of CSases 2S and 3S were confirmed to be identical with those of CSases 2 and 3 (data not shown). Although the *N*-terminal sequence of CSase 1' was shifted to superpose upon *N*-terminal sequences of CSases 2 and 3, the same sequence was not recognized.

Discussion

The plural types of CSase have been found and

Table II. Amino Acid Composition of Cysteine Synthase Isozymes Purified from Spinach Leaves and Hydrated Seeds. CSase 1' was purified from hydrated seeds, and the other CSase isozymes were purified from leaves.

Amino acid	Residues/subunit molecular mass, mol/mol			
	CSase 1	CSase 2	CSase 3	CSase 1'
Asx	27	24	26	24
Thr	18	16	19	13
Ser	25	27	28	36
Glx	37	38	37	39
Pro	19	20	20	7
Gly	35	37	36	58
Ala	29	30	28	20
Val	25	27	25	18
Met	13	13	10	6
Ile	22	26	25	17
Leu	24	25	24	22
Tyr	10	9	8	11
Phe	13	10	9	13
Lys	29	23	24	18
His	5	2	2	10
Arg	9	9	10	17
Total	340	336	331	329

Tryptophan and cysteine could not be assayed.

Table III. Composition of the *N*-terminal Amino Acids of Cysteine Synthase Isozymes from spinach

	1	5	10
CSase 1	Ala-Val-Ser-Leu-Ser-Pro-Pro-Ser-Thr-Ile-		
CSase 2	Ala-Phe-Ile-Ala-Lys-Asp-Val-Thr-Glu-Leu-		
CSase 3	Thr-Val-Thr-Asn-Ala-Lys-Asn-Val-Asp-Gln-		
CSase 1'	Thr-Met-Leu-Lys-Tyr-Lys-Ile-Ala-Val-Phe-		
	11	15	20
CSase 1	Glu-Gly-Leu-Asn-Ile-Ala-Glu-Asp-Val-Ser-		
CSase 2	Ile-Gly-Lys-Thr-Pro-Leu-Val-Tyr-Leu-Asn-		
CSase 3	Leu-Ile-Asp-Arg-Thr-Pro-Lys-Leu-Tyr-Asp-		
CSase 1'	Val-Gln-Lys-Ala-Asn-Asp-Ile-Glu-Ala-Lys-		
	21	25	30
CSase 1'	Leu-Asn-Tyr-Asn-Lys-Ile-Asp-Gly-Ser-Asp-		

purified from several species of plants.⁵⁻¹⁵ From spinach leaves, three types of CSase (CSases 1-3) were purified and the properties of each isozyme were reported.²¹ Furthermore, the nucleotide sequences of cDNA encoding three types of CSase (CSases A-C) were determined for each corresponding enzyme.¹⁷⁻²⁰ With respect to the localization of CSase isozymes (CSases A-C), it was reported that these isozymes were localized in chloroplasts, mitochondria and cytosol.¹⁶ It was decided that CSase 1 localized in chloroplasts corresponding CSase B. On the other hand, the correspondence of CSases 2 and 3 to CSases A and C was not determined.

This paper presents the purification and characterization of a novel isozyme of cysteine synthase, CSase 1', from spinach hydrated seeds. Moreover, according to the determination of *N*-terminal amino acid sequences from all of isozymes, CSases 1, 2 and 3 were correlated with each of CSases A-C reported¹⁷⁻²⁰, respectively (Fig. 7). It is disclosed that CSases 1, 2, and 3 are localized in chloroplasts, cytosol, and mitochondria, respectively.

We had already reported the existence of three CSase isozymes designated as CSases 1', 2S, and 3S with reference to the mobility in hydrated seeds.²² In this report, each isozyme can be separated from each other by disc electrophoresis. For CSases 2S and 3S from hydrated seeds, two isozymes have the same properties as CSases 2 and 3 from spinach leaves²¹ with respect to molecular weight, subunit composition, K_m values for OAS and sulfide, heat stability, immunotitration, pH optimum, and effect of hydroxylamine. From these results, it comes to conclusion that CSases 2S and 3S from hydrated seeds are the same isozymes as CSases 2 and 3 from mature leaves, respectively. CSases 2 and 3 are usually expressed on all of the growing stage from seeds to mature leaves.²²

On the other hand, CSase 1' was only detected during the initial period of germination.²² CSase 1' was first purified from hydrated seeds. The molecular weight of CSase 1' was estimated to be about 68,000, which molecular mass was higher than those of other CSases (63,000). The subunit structure of CSase 1' was a homodimer with identical subunit molecular mass of 34,000, similar to those of other CSases. The specific activity of CSase 1' was 834 units/mg protein, which was

Fig. 7. Amino Acid Alignment of CSase Sequences.

Each amino acid sequence of CSases 1, 2, and 3 was compared with the corresponding amino acid sequences deduced from the cDNA of CSases B,^{18,19} A¹⁷ and C,²⁰ respectively. Identified amino acids sequences indicate in boxed letters, and gaps are indicated by dashes.

higher than that of CSase 1 (400 units/mg protein) but lower than those of CSases 2 and 3 from both hydrated seeds and leaves.²¹ CSase 1' had properties similar to those of the other isozymes with respect to double immunodiffusion analysis, K_m values for OAS and sulfide, and heat stability. However, in particular, the primary structure, effect of immunotitration, pH optimum, and effect of hydroxylamine on CSase 1' were different from those for other CSases. Based on the results of amino acid composition and *N*-terminal sequence, it was concluded that CSase 1' purified from hydrated seeds is a novel isozyme different from other CSases 1, 2, and 3 from leaves.

It has been reported that CSase exists as a complexed form with SATase.²⁹⁻³³ However, the type of CSase isozyme in the complex is unidentified. We investigated the form of CSase complexed with SATase in spinach leaves, finding that all of CSase isozymes bound with SATase, although each CSase isozyme has a different tightness of binding constants (Zhu *et al.*, unpublished results).

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References

- 1) J. W. Anderson, "Sulfur in Biology", University Park Press, Baltimore, 1978.
- 2) N. M. Kredich and G. M. Tomkins, *J. Biol. Chem.*, **241**, 4955-4965 (1966).
- 3) I. K. Smith and J. F. Thompson, *Biochim. Biophys. Acta*, **227**, 288-295 (1971).
- 4) I. K. Smith, *Plant Physiol.*, **50**, 477-479 (1972).
- 5) M. Masada, K. Fukushima, and G. Tamura, *J. Biochem.*, **77**, 1107-1115 (1975).
- 6) K. Nakamura and G. Tamura, *Agric. Biol. Chem.*, **53**, 2537-2538 (1989).
- 7) T. Kanno, Y. Maruoka, T. Matsubayashi, K. Shimpo, M. Masada, and G. Tamura, *Nippon Nogeikagaku Kaishi* (in Japanese), **67**, 15-22 (1993).
- 8) F. Ikegami, M. Kaneko, F. Lambein, Y. H. Kuo, and I. Murakoshi, *Phytochemistry*, **26**, 2699-2704 (1987).
- 9) F. Ikegami, M. Kaneko, H. Kamiyama, and I. Murakoshi, *Phytochemistry*, **27**, 697-701 (1988).

- 10) C. R. Kuske, L. O. Ticknor, E. Guzmán, L. R. Gurley, J. G. Valdez, M. E. Thompson, and P. J. Jackson, *J. Biol. Chem.*, **269**, 6223–6232 (1994).
- 11) H. Fankhauser and C. Brunold, *Plant Sci. Lett.*, **14**, 185–192 (1979).
- 12) B. L. Bertagnolli and R. T. Wedding, *Plant Physiol.*, **60**, 115–121 (1977).
- 13) W. Diessner and A. Schmidt, *Z. Pflanzenphysiol.*, **102**, 57–68 (1981).
- 14) I. Murakoshi, M. Kaneko, C. Koide, and F. Ikegami, *Phytochemistry*, **25**, 2759–2763 (1986).
- 15) M. Droux, J. Martin, P. Sajus, and R. Douce, *Arch. Biochem. Biophys.*, **295**, 379–390 (1992).
- 16) J. E. Lunn, M. Droux, J. Martin, and R. Douce, *Plant Physiol.*, **94**, 1345–1352 (1990).
- 17) K. Saito, N. Miura, M. Yamazaki, H. Hirano, and I. Murakoshi, *Proc. Natl. Acad. Sci. USA*, **89**, 8078–8082 (1992).
- 18) K. Saito, K. Tatsuguchi, I. Murakoshi, and H. Hirano, *FEBS Lett.*, **324**, 247–252 (1993).
- 19) N. Rolland, M. Droux, M. Lebrun, and R. Douce, *Arch. Biochem. Biophys.*, **300**, 213–222 (1993).
- 20) K. Saito, K. Tatsuguchi, Y. Takagi, and I. Murakoshi, *J. Biol. Chem.*, **269**, 28187–28192 (1994).
- 21) T. Yamaguchi and M. Masada, *Biochim. Biophys. Acta*, **1251**, 91–98 (1995).
- 22) S. Kitamura, T. Yamaguchi, X. Zhu, and M. Masada, *Biosci. Biotech. Biochem.*, **60**, 1608–1611 (1996).
- 23) S. Nagai and M. Flavin, *J. Biol. Chem.*, **242**, 3884–3895 (1967).
- 24) M. K. Gaitonde, *Biochem. J.*, **104**, 627–633 (1967).
- 25) O. Ouchterlony, *Progr. Allergy*, **6**, 30–154 (1962).
- 26) M. M. Bradford, *Anal. Biochem.*, **72**, 248–254 (1976).
- 27) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404–427 (1964).
- 28) U. K. Laemmli, *Nature*, **227**, 680–685 (1970).
- 29) N. M. Kredich, M. A. Becker, and G. M. Tomkins, *J. Biol. Chem.*, **244**, 2428–2439 (1969).
- 30) P. F. Cook and R. T. Wedding, *Arch. Biochem. Biophys.*, **178**, 293–302 (1977).
- 31) P. A. Baecker and R. T. Wedding, *Anal. Biochem.*, **102**, 16–21 (1980).
- 32) K. Nakamura, A. Hayama, M. Masada, K. Fukushima, and G. Tamura, *Plant Cell Physiol.*, **29**, 689–693 (1988).
- 33) K. Nakamura and G. Tamura, *Agric. Biol. Chem.*, **54**, 649–656 (1990).