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Effects of Constituent Amino Acids of Glutathione and Ammonium Sulfate added to Hydroponic Solution on the Synthesis of Glutathione in Lettuce

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Abstract

The effects of constituent amino acids of glutathione (GSH), glutamate (Glu), cysteine (Cys) and glycine (Gly), on GSH synthesis in lettuce seedlings were examined in this study. The GSH concentration of the seedlings was increased to 5.1-fold and 1.6-fold the concentration of the control in the first leaves and roots, respectively, by simultaneous application of these constituent amino acids (Glu+Cys+Gly) at 100 mg/l to the culture solution for two days. In the first leaves and roots of these seedlings, the concentration of GSH was 180.4 and 14.6 nmole/gFW, and non-essential amino acids including Glu, Cys and Gly occupied 93.2% and 84.0% of the total free amino acids, respectively. Application of Cys greatly increased the concentration of GSH in the roots, and application of 50 mg/l Cys increased it to 26.1-fold the concentration in the control. The activity of GSH synthetase was higher in the leaves than in the roots, whereas the activity of γ-glutamylcysteine synthetase was higher in the roots than in the leaves.

Key words: Lettuce, glutathione, cysteine, γ-glutamylcysteine synthetase, glutathione synthetase

Introduction

Lettuce (*Lactuca savita* L.) is very popular salad vegetables in Korea. In order to produce lettuce of superior quality containing glutathione (GSH) at a high concentration, we added the constituent amino acids of GSH or ammonium sulfate to hydroponic culture solution, and examined the concentrations of GSH and its precursors in the seedlings.

GSH plays several roles in plant defense systems. In addition to the function as an antioxidant, which may be involved in the redox balance of the cells (Kunert and Foyer 1993), it is effective in the detoxification of xenobiotics and heavy metals (Rennenberg 1982; Alscher 1989). It is a cofactor of some enzymes and of DNA synthesis, and plays a central role in the metabolism of reduced sulfur (Schmidt and Kunert 1986). Therefore, it is of interest to study the factors that enhance the capacity of producing or metabolizing GSH in vegetables.

GSH is a tripeptide consisting of L-glutamate (Glu), L-cysteine (Cys) and glycine (Gly), and synthesized through adenosine triphosphate (ATP)-dependent two consecutive steps. In the first step, dipeptide γ-EC is produced from Glu and Cys catalyzed by γ-glutamylcysteine synthetase (E.C. 6.3.2.2, γ-ECS) through ATP-dependent reaction (Lancaster et al. 1989; Hell and Bergmann 1990; Ruegsegger and Brunold 1992). In the second step, Gly is added to the c-terminal site of the dipeptide (γ-EC) to yield GSH; this reaction is catalyzed by GSH synthetase (E.C. 6.3.2.3, GSHS), also through ATP-dependent reaction (Law and Halliwell 1986; Klapheck et al. 1987; Macnicol 1987; Hell and Bergmann 1988; Rüegsegger et al. 1990).

In Japan, research on fermentative and enzymatic production of GSH for food additives, supplements, and medicines was very active from 1976 to 1985, and fermentative products of GSH by yeast were commercialized in the early 1980's (Li et al. 2004). Much of the technology for

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the production of GSH has been patented. Several materials were found to have a stimulatory effect on GSH production: wastewater in yeast (Asai and Kume 1943), amino acids in *Lactococcus lactis* (Li et al. 2005), amino acids in *Rhodotorula glutinis* (Cho et al. 1978), amino acids in *Escherichia coli* (Li et al. 1998), anisomycin in *Saccharomyces cerevisiae* (Watanabe 1973) and amino acids in *Saccharomyces cerevisiae* (Kusakabe 1973; Ooka 1973a,b; Mimura 1973; Wen et al. 2004).

The underlying control mechanisms leading to up-regulation of GSH synthesis in plants have not been fully defined. In order to obtain more information about GSH synthesis in higher plants, in particular to elucidate the effect of additives, such as constituent amino acids on GSH production in lettuce (*Lactuca sativa* cv. Sisuko), we examined the concentrations of GSH, γ-EC and cysteine in the seedlings under hydroponic culture supplemented with constituent amino acids of GSH.

Material and Methods

Plant material and Treatment

Seeds of lettuce (*Lactuca sativa* cv. Sisuko) were germinated and grown for seven days in cell flats (cell size, 3 cm × 3 cm × 10 cm) filled with vermiculite, and the flats were transferred to a cultivation chamber under controlled environmental conditions with a relative humidity of 60-80 % at 25℃/15℃ (day/night) and 16 h/8 h photoperiod. The composition of the culture solution (in mg/l) was 40 N (NO_3^- : NH_4^+=1:1), 40 P_2O_5, 40 CaO, 10 MgO, 5 Fe_2O_3, 0.5 MnO, 0.4 B, 0.05 Mo, 0.02 Cu and 0.05 Zn, used by Ohta et al. (1970). The nutrient solution (pH 5.5-6.0) was renewed every two days and aerated continuously. Seven-day-old seedlings uniform in size were transplanted to the nutrient solution in aerated tanks (2.9 l volume), and grown until the second leaves had fully expanded (seven days after transplanting). Then a constituent amino acid of GSH (Glu, Cys, Gly) alone or in combination, or ammonium sulfate was added to the solution at the concentration of 0 (control), 50 or 100 mg/l. Plants were harvested after 48 h treatments were initiated, the roots and the first leaves were isolated, and they were immediately frozen in liquid nitrogen and stored at -80℃ until use for analysis.

Extraction and preparation of thiol derivatives of GSH, γ-EC and Cys

GSH, γ-EC and Cys in the plant extracts were converted to thiol derivatives as described by Kocsy et al. (2000). The plant material was ground with liquid nitrogen in a mortar, then 5 ml of 0.1 M HCl containing 1 mM Na_2EDTA was added to 500 mg of the sample. After mixing, the solution was centrifuged at 16,000 ×g for 15 min at 4℃, 400 μl of the supernatant was added to 600 μl of 0.2 M 2-[cyclohexylamino] ethane sulphonic acid (pH 9.3), and it was reduced with 100 μl of a freshly prepared 400 mM $NaBH_4$ solution. The mixture was kept on ice for 20 min, and 15 μl of 15 mM monobromobimane (Fahey and Newton 1987) was added to 330 μl of the mixture. Then the mixture was kept in the dark at room temperature for 15 min to obtain thiol derivatives. The reaction was stopped by adding 250 μl of 5% (v/v) acetic acid and the reactant was centrifuged at 16,000 ×g for 15 min at 4℃.

Determination of free amino acids

Free amino acids were extracted according to Desmaison et al. (1984) with minor modifications. Frozen samples were ground into a fine powder in liquid nitrogen with a chilled mortar and a pestle and 300-500 mg of the sample was homogenized in 2 ml of 15 mM HCl. The homogenate was centrifuged at 2,000 ×g for 5 min at 4 ℃, and 500 μl of the supernatant was deproteinized with 100 μl of 5-sulfosalicylic acid (10% w/v) and kept on ice for 15 min. The mixture was again centrifuged at 2,000 ×g for 15 min at 4 ℃. The supernatant (500 μl) was collected and adjusted to "pH 2.2-2.3". The concentrations of free amino acids were measured with an amino acid analyzer (JEOL, JLC-300, Japan) in comparison with mixed amino acid standard solutions (AN and B types (Wako Pure Chemical)).

Assay of γ-ECS and GSHS activities

Activities of γ-glutamylcysteine synthetase (γ-ECS) and GSH synthetase (GSHS) were assayed by the method described by Hell and Bergmann (1990) with some modification. In a chilled mortar, 0.5 g of frozen samples were ground together with 10 mL of extraction buffer (0.1 M Tris-HCl, pH 7.5, 5 mM EDTA) and 1 g of polyvinylpolypyrolidone (Sigma). The slurry was centrifuged at 15,000 ×g for 15 min at 4℃ and the supernatant was used to determine the activities of γ-ECS and GSHS.

For the assay of γ-ECS, a mixture of 100 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 1 mM DTT, 10 mM ATP, 50 mM Na-L-glutamate and 285 μl of protein extract in a total volume of 500 μl were pre-incubated in a tube at 37℃ for 10 min, and then 2 mM L-cysteine (Cys) was added to the mixture. After another 45-min incubation, the reaction was stopped by adding 50 μl of 50% (w/v) 5-sulfosalicylic acid, and the tube was transferred onto the ice. Denatured pro-

teins were removed by centrifugation and the supernatants were assayed for γ-ECS by HPLC. The quantity of γ-ECS synthesized was determined by comparison with the peak area of authentic γ-ECS (Nacalai Tesque, Kyoto, Japan). The mixture without the addition of Cys was also assayed as the control.

For the assay of GSHS, a mixture of 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 5 mM creatine phosphate, 2 units of creatine kinase, 1.5 mM γ-EC and 295 μl of protein extract in a total volume of 500 μl, were pre-incubated at 30℃ for 10 min, and then 10 mM glycine (Gly) was added to the mixture. After additional 30-min incubation, the reaction was terminated and subjected to HPLC analysis as described for the γ-ECS assay. The quantity of GSH synthesized was determined by HPLC analysis. The mixture without the addition of Gly was also assayed as the control.

Protein content assay

The protein contcentration was measured in the leaf and root extracts using the standard Biorad Coomassie brilliant blue assay as described by Bradford (1976).

HPLC of thiol derivatives

An LC-6A HPLC system (Shimadzu, Kyoto, Japan) equipped with a SPD-6A detector, a SCL-6A system controller and a C-R8A data module was used. An Intersil ODS-2(r) column 5 C_{18} (150×4.6mm, 5microm) (GC Science Inc, Tokyo, Japan) was used. Mobile phase A contained 0.1% TFA in water and mobile phase B was MeOH, complementary to the TFA solution (A%=100%-B%). A gradient elution using MeOH was performed for better analyte separation and column cleansing prior to subsequent injections. For analysis of GSH, γ-EC and cysteine, the mobile phase system was programmed as follows: 0-30 min, 15-22% B; 30-35 min, 22-100%B; 35-40 min, isocratic 100%B; 40-45 min, 100-15%B, 45-50 min, isocratic 15%B. The chromatography was operated at 30℃ using a flow rate of 0.8 ml/min. The fluorescence detector was set at 380 nm for the excitation and 480 nm for the emission mode.

Chemicals

Glutathione (GSH), glutathione disulfide (GSSG), diethyl ether, Glu, Gly and Cys were purchased from Wako Pure Chemicals (Osaka, Japan). Glutathione reductase (E.C. 1.6.4.2, GR) and γ-EC were purchased from Oriental Yeast (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) from Kanto Chemicals (Tokyo, Japan). Monobromobimane (mBBr) was purchased from Calbiochem (San Diego, Calif.). All other reagents used for the study were of the highest quality available.

Results

Effects of the constituent amino acids on GSH biosynthesis

The hydroponic culture solution was supplemented with constituent amino acids of GSH (Glu, Cys, Gly) singly or in combination, and the concentrations of Cys, γ-EC and GSH in the first leaf (simply called leaves, hereafter) and root of lettuce seedlings were examined. In the control without any constituent amino acids added, the concentrations of GSH and γ-EC in the leaves were 21.1 + 2.1 and 1.2 + 0.0 nmole/gFW, respectively, and those in the roots were 9.2 + 0.4 and 0.3 + 0.0 nmole/gFW, respectively (Table 1).

Glu, Cys and Gly added singly to the hydroponic solution increased the concentrations of GSH both in the leaves and roots, and Cys was particularly effective in increasing the production of GSH in the roots (Table 1). The concentration of γ-EC was not increased by Glu and Gly, but was greatly increased by Cys.

Combined application of Glu, Cys and Gly (Glu+Cys+Gly) at 50 or 100 mg/l to the hydroponic culture solution increased the concentration of GSH in the leaves to 1.6-5.1-fold the concentration in the control. It also increased the concentration of GSH in the roots to 1.0-1.6-fold of the control (Table 1). Glu+Cys, Cys+Gly and Glu+Gly also increased the concentration of GSH in both leaves and roots. Glu+Cys and Cys+Gly increased the concentrations of Cys and γ-EC in both leaves and roots.

Application of Glu+Cys+Gly amino acid at 50 and 100 mg/l increased the activity of GSHS in the leaves to about 2.0-fold and 1.8-fold the activity in the control, respectively, and the activity of γ-ECS in the leaves to about 1.8-fold and 3.0-fold the activity in the control, respectively. The increased activity of γ-ECS may account for the increased concentration of GSH. The activities of GSHS and γ-ECS in the roots were also increased by Glu+Cys+Gly similarly (Table 4).

Exogenous application of the intermediates at early steps of sulfur assimilation pathway, such as sulfide and SO_3^{2-}, are known to be toxic to cells (Andreas and Mark 2002). Thus, we examined the effect of ammonium sulfate $(NH_4)_2SO_4$ at 50 and 100 mg/l on the concentration of Cys, γ-EC and GSH in the leaves and roots. Application of (NH4)2SO4 to the culture solution (50 and 100 mg/l) had significant effect

Table 1. Effects of the constituent amino acids (Glu, Cys, Gly) added to hydroponic culture solution on the concentration of Cys, γ-EC and GSH in the first leaf and root of lettuce.

		Cys		ã-EC		GSH	
		1st leaf	Root	1st leaf	Root	1st leaf	Root
Control		8.9±0.5	12.2±0.3	1.2±0.0	0.3±0.0	21.1±2.1	9.2±0.4
Glu	50 mg/l	10.5±0.5	20.9±0.3	0.5±0.0	0.3±0.0	55.5±3.3	27.6±1.7
	100 mg/l	11.9±1.2	16.5±0.1	1.5±0.1	0.4±0.0	100.7±3.0	44.2±2.4
Cys	50 mg/l	247.7±13.0	142.3±9.9	4.1±0.2	5.7±0.3	29.8±1.2	239.7±15.0
	100 mg/l	426.7±39.4	77.7±6.7	12.2±1.3	4.1±0.1	72.4±3.4	219.5±13.8
Gly	50 mg/l	8.9±0.2	16.3±0.5	0.7±0.0	0.5±0.0	24.2±0.3	21.9±0.3
	100 mg/l	10.3±0.9	13.5±0.1	1.1±0.1	0.7±0.0	25.6±2.1	27.4±2.8
Glu+Cys	50 mg/l	87.7±7.3	35.7±2.2	6.4±0.2	1.3±0.1	22.6±1.2	7.9±0.6
	100 mg/l	248.1±17.1	37.1±0.3	6.5±0.6	1.8±0.1	64.3±0.4	21.6±0.9
Cys+Gly	50 mg/l	59.8±1.0	19.2±0.6	9.0±0.8	2.2±0.1	64.5±4.2	27.7±0.1
	100 mg/l	96.2±4.0	40.9±2.0	14.2±1.8	2.3±0.2	100.1±4.8	31.7±1.3
Glu+Gly	50 mg/l	37.4±0.1	19.8±1.7	1.0±0.0	0.4±0.0	18.6±0.5	26.7±0.6
	100 mg/l	16.1±1.1	30.0±0.7	0.5±0.0	0.3±0.0	45.1±1.7	36.6±2.2
Glu+Cys+Gly	50 mg/l	16.1±1.2	29.3±1.5	2.5±0.0	0.5±0.0	33.6±0.9	9.2±0.4
	100 mg/l	40.6±1.2	31.0±0.6	11.4±0.7	0.5±0.1	108.4±4.7	14.6±1.2

All values are means + SE of five independent extractions. Concentrations are shown as nmol per g fresh weight.

on the concentrations of Cys, γ-EC and GSH, especially, $(NH_4)_2SO_4$ at 100 mg/l increased the concentration of GSH in the leaves (Table 5). We infer from this result that ammonium sulfate does not suppress GSH biosynthesis and rather promote it at a high concentration (100 mg/l)

Effects of the constituent amino acids on the concentrations of free amino acids and ammonia in lettuce

Table 2, 3 shows the effects of Glu+Cys and Glu+Cys+Gly on the concentrations of free amino acids, substrates of GSH synthesis, in the shoots and roots of lettuce seedlings. Free amino acid concentration increased when added amino acid (Glu+Cys and Glu+Cys+Gly) availability was increased, being higher in roots than in shoots.

The concentration of total amino acids (TAA), especially non-essential amino acids (NEAA), in the shoots was increased by the treatment with Glu+Cys+Gly (2.0-fold). In the control seedlings the concentration of total EAA was only 6.7 (26)% of TAA and that of total NEAA occupied 93.3 (74)% of TAA. In the seedlings supplied with Glu+Cys+Gly at 100 mg/l, NEAA and EAA occupied 93.2 (84)% and 6.8 (16)% of TAA, respectively (root).

Among the EAA in the shoots, the concentrations of lysine, and among the NEAA in the shoots, the concentrations of proline were decreased by Glu+Cys+Gly at 100 mg/l, though the concentrations of other amino acids were increased. Isoleucine, leucine and valine quantitatively dominated (63%) the EAA pool, while aspartic acid, glutamic acid and glutamine quantitatively dominated the non-essential amino acid (NEAA) pool (66%) by the treatment with Glu+Cys+Gly 100 mg/l (Table 2).

In the roots also, the concentration of TAA was increased by the addition of Glu+Cys (1.1-1.3 fold) and Glu+Cys+Gly (1.5-2.1 fold), but the concentration of lysine, threonin (EAA) and cystine (NEAA) was decreased by the addition of Glu+Cys+Gly at 100 mg/l. Isoleucine, leucine and valine quantitatively dominated (56%) the EAA pool, while glutamine and serine quantitatively dominated the non-essential amino acid (NEAA) pool (70%) by the treatment with Glu+Cys+Gly 100 mg/l (Table 3).

Interestingly, in the shoots, methionine and tryptophan, and in the roots, tryptophan were not detected. The concentration of TAA and protein was increased by the treatment with Glu+Cys and Glu+Cys+Gly. The concentration of NH_3 was extremely higher in the roots than in the shoots. However, Britto and Kronzucker (2002) already demonstrated that the allocation of carbon to the root for amino acid synthesis under NH_4^+ nutrition is higher than that to the leaf. The concentration of ammonia was greatly decreased by the treatment with Glu+Cys and Glu+Cys+Gly in

Table 2. Concentration of free amino acids in the first leaf of lettuce supplied with Glu+Cys+Gly.

1st leaf	Control	Glu+Cys (50 mg/l)	Glu+Cys (100 mg/l)	Glu+Cys+Gly (50 mg/l)	Glu+Cys+Gly (100 mg/l)
Arginine	10.9±0.6	6.3±3.9	8.1±0.5	12.9±2.1	25.8±2.7
Histidine	5.3±0.2	5.6±0.6	7.4±1.1	6.4±0.3	12.3±0.6
Isoleucine	17.3±1.8	15.9±2.2	16.9±2.0	17.9±2.4	39.7±1.2
Leucine	15.2±0.8	15.4±0.6	16.2±1.4	13.8±1.6	27.0±0.4
Lysine	4.0±0.1	n.d.	0.5±1.0	1.9±0.6	3.5±0.0
Methionine	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylalanine	13.3±0.5	13.1±0.4	13.1±0.6	12.1±1.0	20.4±1.0
Threonin	3.9±0.4	3.6±0.5	2.9±0.3	2.4±0.4	9.2±1.1
Tryptophan	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	25.1±0.8	21.4±2.7	28.2±2.1	28.7±1.6	55.6±0.6
EAA	95.2±5.2	81.3±10.9	93.4±9.0	96.0±10.0	193.5±7.7
Alanine	136.8±5.8	113.9±11.4	163.3±4.4	154.1±1.6	235.4±6.2
Asparagine	n.d.	5.6±2.1	10.8±2.8	8.0±0.8	26.4±2.5
Aspartic acid	299.8±11.6	368.0±17.6	434.1±14.7	366.3±6.4	448.2±7.3
Cystine	n.d.	2.4±0.5	4.0±0.0	4.0±1.2	19.8±0.6
Glutamic acid	651.9±10.6	720.9±8.6	732.1±15.2	635.3±8.4	683.2±13.2
Glutamine	73.7±5.2	37.9±3.0	137.1±4.6	146.8±11.7	617.6±71.7
Glycine	16.9±1.1	11.7±1.2	16.3±3.1	18.9±2.5	142.0±24.2
Proline	12.2±2.3	10.9±1.1	11.1±0.7	8.5±0.7	11.3±0.6
Serine	95.2±2.2	71.8±8.3	97.0±14.7	107.7±9.7	372.2±44.9
Tyrosine	38.5±0.5	36.8±4.1	44.1±3.9	43.9±2.2	78.6±2.1
NEAA	1324.9±39.3	1379.9±57.9	1649.8±64.1	1493.4±45.2	2634.7±173.1
TAA	1420.1±44.5	1461.2±68.8	1743.2±73.1	1589.4±55.2	2828.1±180.9
E/T	6.7	5.6	5.4	6	6.8
NE/T	93.3	94.4	94.6	94	93.2
Protein comtent	3.8±1.0	4.5±0.3	3.9±0.3	3.0±0.4	3.8±0.5
Urea	40.4±3.2	40.0±6.3	40.9±10.4	32.0±9.6	26.8±1.9
Amonia	189.4±6.7	228.4±42.1	243.5±30.7	292.6±46.5	429.9±50.3

Free amino concentrations are shown as umol per g fresh weight. All values are means + SE of five independent extractions. n.d.: not detected

the root, but it was increased in the shoots. The concentration of glutamine increased more than that of the other amino acids. As a result, glutamine in percentage of total free amino acids was not constant, but it increased 5-22 and 29-43% with increasing concentrations of free amino acids in shoots and roots, respectively. The concentration of urea was also extremely decreased by the treatment with Glu+Cys+Gly in the shoots, but it was slightly increased.

Discussion

In environmental conditions, stresses or chemicals have been reported to increase GSH contents in plants (Kopriva and Rennenberg 2004; Tausz et al. 2004; Hirase and Molin, 2003). To increase the synthesis of GSH in lettuce, we added the three constituent amino acids of GSH, Glu, Cys and Gly, to the culture medium. All of these amino acids promoted GSH synthesis, and the same has been reported in *E. coli* (Li et al. 1998). Combined application of Glu, Cys and Gly (Glu+Cys+Gly) was most effective in increasing the concentration of GSH in the leaves. Cys at 100 mg/l increased the concentration of GSH in the leaves to 3.4- fold (Table 1). However, Cys was extremely effective in increasing the concentration of GSH in the roots, and the concentration of GSH in the roots of the seedlings supplied with Glu+Cys+Gly at 50 mg/l was about 26-folds than the

Table 3. Concentration of free amino acids in the roots of lettuce supplied with Glu+Cys+Gly.

Root	Control	Glu+Cys (50 mg/l)	Glu+Cys (100 mg/l)	Glu+Cys+Gly (50 mg/l)	Glu+Cys+Gly (100 mg/l)
Arginine	73.0±2.2	98.5±4.2	93.7±5.7	85.6±3.5	87.5±2.9
Histidine	58.8±3.4	46.4±4.2	55.2±0.8	58.7±0.3	58.3±3.9
Isoleucine	87.0±1.8	80.4±5.2	75.8±2.7	84.1±3.2	93.6±2.5
Leucine	68.4±2.0	89.1±8.5	93.0±3.3	76.1±3.4	110.5±4.7
Lysine	44.3±2.5	37.0±1.7	34.7±1.2	31.0±2.6	30.0±1.2
Methionine	n.d.	n.d.	2.6±0.2	2.0±0.1	2.0±0.2
Phenylalanine	45.8±2.5	52.5±4.1	65.5±1.3	60.9±3.6	68.9±2.1
Threonin	59.1±2.8	37.5±4.5	46.7±1.7	48.9±1.2	54.6±3.3
Tryptophan	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	94.7±0.5	126.6±9.8	146.8±2.2	120.8±5.3	173.2±3.4
EAA	531.2±17.9	568.0±42.2	614.1±19.0	568.0±23.2	678.6±24.3
Alanine	110.2±2.1	156.1±23.5	210.2±11.2	138.8±12.9	176.0±12.3
Asparagine	48.2±1.5	73.0±3.8	57.1±2.6	85.4±4.3	95.6±4.6
Aspartic acid	81.0±5.5	100.6±5.5	114.6±4.9	105.0±2.2	119.7±3.9
Cystine	4.8±0.5	2.3±0.6	4.0±0.4	1.7±0.3	3.1±0.4
Glutamic acid	310.9±11.7	189.8±16.8	265.0±4.3	285.3±4.7	310.5±8.6
Glutamine	588.0±32.5	678.2±35.4	892.5±55.8	1171.8±54.8	1808.3±35.5
Glycine	28.9±0.9	46.6±5.0	54.7±0.9	81.5±7.5	165.4±2.8
Proline	31.9±0.6	35.2±5.6	35.4±0.4	27.5±0.7	32.7±6.1
Serine	184.9±4.1	224.8±18.8	281.6±5.7	388.7±38.3	689.8±8.1
Tyrosine	119.1±2.6	120.8±6.3	129.4±2.0	127.3±4.8	149.2±4.9
NEAA	1507.9±61.9	1627.3±121.3	2044.4±88.1	2413.0±130.5	3550.2±87.3
TAA	2039.1±79.8	2195.3±163.5	2658.5±107.1	2981.1±153.7	4228.8±111.6
E/T	26	25.9	23.1	19.1	16
NE/T	74	74.1	76.9	80.9	84
Protein comtent	5.5±1.1	6.4±0.4	6.1±0.6	8.2±0.6	10.5±1.8
Urea	126.9±11.7	418.0±30.1	162.1±7.5	214.0±2.9	129.1±18.4
Amonia	3976.1±76.4	2380.6±130.5	1917.3±26.2	2188.2±55.4	2781.5±122.2

Free amino concentrations are shown as umol per g fresh weight. All values are means + SE of five independent extractions.
n.d.; not detected

Table 4. Effect of Glu+Cys+Gly added to hydroponic culture solution on the enzymes for GSH synthesis in lettuce seedlings. GSHS and γ-ECS were assayed in extracts from lettuce seedlings 2 d after the addition of Glu+Cys and Glu+Cys+Gly. Results are means ± (SE) of five independent extractions from the 1st leaf and root. Enzyme activities are shown as nmol per min per g protein.

Crude extract	GSHS		γ-ECS	
	1st leaf	root	1st leaf	root
Con	6.60±0.11	2.81±0.21	0.04±0.01	0.04±0.00
Glu+Cys(50 mg/l)	8.12±0.22	2.39±0.04	0.09±0.00	0.23±0.00
Glu+Cys(100 mg/l)	8.08±0.53	2.74±0.04	0.08±0.00	0.45±0.01
Glu+Cys+Gly (50 mg/l)	13.08±0.42	4.33±0.25	0.07±0.02	0.13±0.02
Glu+Cys+Gly (100 mg/l)	11.67±0.94	4.97±0.13	0.12±0.01	0.26±0.02

Table 5. Effect of ammonium sulfate on the concentrations of Cys, γ-EC and GSH in the first leaf and root of lettuce seedling. 50 mg/l and 100 mg/l $(NH_4)SO_4$ are equivalent to 0.38 mM and 0.76 mM, respectively. All values are means ± SE of five independent extractions.

$(NH_4)SO_4$	Cys		γ-EC		GSH	
	1st leaf	Root	1st leaf	Root	1st leaf	Root
Con	8.9±0.5	12.2±0.3	1.2±0.0	0.3±0.0	21.1±2.1	9.2±0.4
0.38 mM	12.6±0.4	18.1±0.6	1.3±0.0	0.4±0.0	23.4±1.4	23.3±1.1
0.76 mM	18.7±0.5	18.1±1.3	2.2±0.1	0.4±0.1	90.4±2.1	15.0±1.4

concentration in the control. A stimulatory effect of Cys on GSH synthesis was observed in poplar (Noctor and Strohm 1996) and in recombinant *E. coli* (Li et al. 1998) in which the total GSH concentration and the intracellular GSH concentration were increased by 40% and 100%, respectively, by application of 9 mM Cys to the culture medium for 12 h. Similar results were also reported by Alfafala et al. (1992).

Factors promoting GSH synthesis have been reported to repress the production of γ-EC (Bergmann and Rennenberg 1993; Schneider and Bergmann 1995) and Cys (Ruegsegger and Brunold 1992; Strohm et al. 1995). In the present experiments also, the concentrations of γ-EC in the leaves of the seedlings supplied with Glu, Gly or Glu+Gly were as low as those in the control, although the concentration of GSH was clearly increased by the addition of these amino acids. However, addition of Cys, Glu+Cys, Cys+Gly or Glu+Cys+Gly markedly increased the concentrations of Cys and γ-EC (Table 1). Application of Cys seems to increase the concentration of Cys and γ-EC, though Glu+Cys+Gly had only a slight effect on the concentrations of Cys and γ-EC. The increase in the constituent amino acids of GSH, especially Cys might have an important role to enhance the enzyme activities for GSH synthesis and GSH concentration in the plants.

A notable characteristics of the lettuce is the high concentration of ammonia in the roots (Table 3). However, nitrate can be stored at low energetic costs in the vacuoles of roots and shoots, but ammonium has to be assimilated immediately upon its influx into the roots to avoid the toxic effects of ammonium accumulation (Cruz et al. 1993). In this study, the concentration of ammonia in the roots was decreased dramatically by the treatment with Glu+Cys and Glu+Cys+Gly, but, was increased in the shoots. The concentration of urea was also decreased by Glu+Cys+Gly in the shoots, but slightly increased in the roots. Thus, the application of Glu+Cys+Gly increased GSH concentration, protein synthesis and decreased the damage by ammonia, which might be partly due to conversion of ammonia to urea.

In conclusion, the GSH concentration of lettuce was increased by supplying constituent amino acids (Glu, Cys, Gly) to the culture solution, and the application of Cys extremely increased the GSH content of the roots. Although more research is necessary, we believe that addition of constituent amino acids of GSH or increase in Cys concentration through sulfur assimilation increases GSH concentration and the nutritional value of lettuce, since GSH is considered as a nutraceutical (functional) foods.

References

Alfafala CG, Kanda A, Shioi T, Shimizu H, Shioya S, Suga K (1992) Effect of amino acids on glutathione production by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 36: 538-540

Alscher RG (1989) Biosynthesis and antioxidant function of glutathione in plants. Physiol Plant 77: 457-464

Andreas JM, Mark DF (2002) Control of demand-driven biosynthesis of glutathione in green Arabidopsis suspension culture cells. Plant Physiol 130: 1927-1937

Asai I, Kume S (1943) Culturing of yeast using vitamin B1 extracted from wastewater from rice bran and its glutathione content. J Agricultural Chemical 19: 275-278 (in Japanese)

Bergmann L, Rennenberg H (1993) Glutathione metabolism in plants. In: De Kok LJ, Stulen I, Rennenberg H, Brunold C, Rauser WE (eds), Sulfur Nutrition and Sulfur Assimilation in Higher Plants. SPB Academic Publishers, The Hague, The Netherlands, pp 109-123

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: a critical review. J Plant Physiol 159: 567-584

Cho WD, Kim HI, Song JC, Yang HC (1978) Studies on the production of glutathione by Microorganism. Kor J Appl Microbiol Bioeng 6: 75-80 (in Korean)

Cruz C, Lips SH, Martins-Loucao MA (1993) Nitrogen assimilation and transport in carob plants. Physiol Plant 89: 524- 531

Desmaison AM, Marcher MH, Tixier M (1984) Changes in the free and total amino acid composition of ripening chestnut seeds. Phytochem 23: 2453-2456

Fahey RC, Newton GL (1987) Determination of low-molecular weight thiols using monobromobimane fluorescent labelling and high performance liquid chromatography. Methods Enzymol 143: 85-96

Hell R, Bergmann L (1988) Glutathione synthetase in tobacco suspension cultures: catalytic properties and localization. Physiol Plant 72: 70-76

Hell R, Bergmann L (1990) γ -Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. Planta 180: 306-312

Hirase K, Molin WT (2003) Sulfur assimilation in plants and weed control: Potential targets for novel herbicides and action sites of certain safeners. Weed Biology and Management 3: 147-157

Klapheck S, Latus C, Bergmann L (1987) Localization of glutathione synthetase and distribution of glutathione in leaf cells of *Pisum sativum* L. J Plant Physiol l31: 123-131

Kocsy G, Ballmoos PV, Suter M, Rüegsegger A, Galli U, Szalai G, Galiba G, Brunold C (2000) Inhibition of glutathione synthesis reduces chilling tolerance in maize. Planta 211: 528-536

Kopriva S, Rennenberg H (2004) Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. J Exp Biol 404: 1831-1842

Kunert KJ, Foyer CH (1993) Thiol/disulphide exchange in plants. In: De Kok LJ, Stulen I, Rennenberg H, Brunhold C, Rausen W (eds), Sulfur Nutrition and Assimilation in Higher Plants. Regulatory, Agricultura1 and Environmental Aspects. SPB Academic, The Hague, pp, 139-151

Kusakabe H (1973) Process of manufacture of glutathione. Japan patent. 48-44487 (in Japanese)

Lancaster JE, Reynolds PHS, Shaw ML, Domisse EM, Munro J (1989) Intra-cellular localization of the biosynthetic pathway to flavour precursors in onion. Phytochem 28: 461-464

Law MY, Halliwell B (1986) Purification and properties of glutathione synthetase from spinach (*Spinacia oleracea*) leaves. Plant Sci 43: 185-191

Li Y, Chen J, Mao YY, Lun SY, Koo YM (1998) Effect of additives and fed-batch culture strategies on the production of glutathione by recombinant, *Escherichia coli*. Process Biochem 33: 709-714

Li Y, Hugenholtz J, Sybesma W, Abee T, Molenaar D (2005) Using *Lactococcus lactis* for glutathione overproduction. Appl Microbiol Biotechnol 67: 83-90

Li Y, Wei G, Chen J (2004) Glutathione: a review on biotechnological production. Appl Microbiol Biotechnol 66: 233-242

Macnicol PK (1987) Homoglutathione and glutathione synthetases of legume seedlings: partia1 purification and substrate specificity. Plant Sci 53: 229-235

Mimura H (1973) Process of manufacture of L-glutathione high content yeast. Japan patent 48-92579 (in Japanese)

Noctor G, Strohm M, Jouanin L, Kunert KJ, Foyer CH, Rennenberg H (1996) Synthesis of glutathione in leaves of transgenic poplar overexpressing γ -glutamylcysteine synthetase. Plant physiol 112: 1071-1078

Ohta Y, Yamamoto K, Deguchi M (1970) Effect of calcium supply and plant age on the distribution of calcium salts in rice. Jpn J Soil Sci Plant Nutr 41: 19-26 (in Japanese)

Ooka T (1973a) Process of manufacture of glutathione by fermentation method. Japan patent 48-44488 (in Japanese)

Ooka T (1973b) Process of manufacture of glutathione by fermentation method. Japan patent 48-61689 (in Japanese)

Rennenberg H (1982) Glutathione metabolism and possible biological roles in higher plants. Phytochem 21: 2778-2781

Ruegsegger A, Brunold C (1992) Effect of cadmium on γ -glutamylcysteine synthesis in maize seedlings. Plant Physiol 99: 428-433

Ruegsegger A, Schmutz D, Brunold C (1990) Regulation of glutathione synthesis by cadmium in *Pisum sativum* L. Plant Physiol 93: 1579-1584

Schmidt G, Kunert KJ (1986) Lipid peroxidation in higher plants. The role of glutathione reductase. Plant Physiol 82: 700-703

Schneider S, Bergmann L (1995) Regulation of glutathione synthesis in suspension cultures of parsley and tobacco. Bot Acta 108: 34-40

Steffens JC (1990) The heavy metal-binding peptides of plants. Annu Rev Plant Physiol Plant Mo1 Biol 41: 553-575

Strohm M, Jouanin L, Kunert KJ, Pruvost C, Polle A, Foyer CH, Rennenberg H (1995) Regulation of glutathione synthesis in leaves of transformed poplar (*Populus tremula x P.alba*) overexpressing glutathione synthetase. Plant J 7: 141-145

Tausz M, ircelj H, Grill D (2004) The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid J Exp Biol 404: 1955-1962

Wallsgrove RM, Keys AJ, Lea PJ, Mifin BJ (1983) Photosynthesis, photorespiration and nitrogen metabolism. Plant Cell Envron 6: 301-309

Watanabe K (1973) Glutathione increase method of yeast. Japan patent 48-40987 (in Japanese)

Wen S, Zhang T, Tan T (2004) Utilization of amino acids to enhance glutathione production in *Saccharomyces cerevisiae*. Enzyme and Microbial Technology 35: 501-507