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# γ-GLUTAMYLTRANSPEPTIDASES IN THE METABOLISM OF γ-GLUTAMYL PEPTIDES IN PLANTS

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**Key Word Index**—*Phaseolus radiatus*; *P. angularis*; *Glycine max*; *Vicia faba*; *Pisum sativum*; Leguminosae, *Asparagus officinalis*; Liliaceae; γ-glutamyltranspeptidase; immature seeds; free amino acids; γ-glutamyl peptides.

**Abstract**—The activity and specificity of γ-glutamyltranspeptidase in immature seeds of some leguminous plants did not reflect the γ-glutamyl peptide pattern in their mature seeds. The γ-glutamyltranspeptidase activity was very low in immature seeds of *Phaseolus radiatus* although the concentration of γ-glutamyl peptides in mature seeds was high. Activity increased during germination while the γ-glutamyl peptides decreased. Inconsistency between specificity of γ-glutamyltranspeptidase and γ-glutamyl peptide pattern was also observed in shoots of *Asparagus officinalis*. γ-Glutamyltranspeptidases may play a role in degradation of γ-glutamyl peptides rather than in their biosynthesis during ripening.

## INTRODUCTION

More than 70 γ-glutamyl peptides have been isolated from plants. Most of them are formed during ripening and accumulated in storage tissues such as seeds or bulbs [1]. It has been proposed that γ-glutamyl peptides found in plants are products of γ-glutamyltranspeptidase (EC 2.3.2.2) activity *in vivo*, and γ-glutamyltranspeptidases have been identified in a number of plants [2–4]. It has not been shown, however, that the activity of γ-glutamyltranspeptidase increases during accumulation of γ-glutamyl peptides, whereas decreasing concentration of γ-glutamyl peptides and increase in γ-glutamyltranspeptidase activity during germination of *Glycine max* has been reported [5]. This paper deals with the comparison of activities and specificities of γ-glutamyltranspeptidases in immature tissues of various plants with different patterns of γ-glutamyl peptides in the mature stage.

## RESULTS AND DISCUSSION

Mature seeds of *Phaseolus radiatus* (*Vigna mungo*) contain considerable amounts of γ-glutamylmethionine and its sulfoxide [6–8]. Mature seeds of *Glycine max* contain γ-glutamylphenylalanine and γ-glutamyltyrosine in high concentrations [9, 10]. A large amount of γ-glutamyl-β-phenyl-β-alanine has been found in mature seeds of *Phaseolus angularis* [11]. The amounts of γ-glutamyl peptides, if any, are very small in mature seeds of *Vicia faba* [12] and *Pisum sativum*. The contents of γ-glutamyl peptides in green shoots of *Asparagus officinalis* are very low [13], although γ-glutamyltranspeptidase activity has been demonstrated and partially purified from this source [14].

Immature seeds of *P. radiatus*, *P. angularis*, *V. faba* and *P. sativum* and green shoots of *A. officinalis* were analysed for γ-glutamyltranspeptidase activity (Table 1). The presence of trace amounts of γ-glutamyl-β-phenyl-β-alanine in *P. angularis* was recognized by 2D-PC and HVE. No γ-glutamyl peptides were detected in immature seeds of *P. radiatus*, *G. max*, *V. faba* and *P. sativum*. A relatively high activity of γ-glutamyltranspeptidase was found only in immature seeds of *G. max* and shoots of *A. officinalis*. The very low activity of γ-glutamyltranspeptidase in immature seeds of *V. faba* and *P. sativum* was consistent with the observation that mature seeds of both legumes contain only trace amounts of γ-glutamyl peptides, if any. However, the activity in immature seeds of *P. radiatus* and *P. angularis*, which contain γ-glutamyl peptides in high concentrations in mature seeds, was also very low. γ-Glutamyltranspeptidase activity in immature seeds of *G. max* was relatively high, but the specificity of the enzyme did not correspond with the pattern of γ-glutamyl peptides in mature seeds as described below (Table 3). Shoots of *A. officinalis*, which contain very low concentrations of γ-glutamyl peptides [13], had high γ-glutamyltranspeptidase activity, in agreement with a previous report [14]. The γ-glutamyltranspeptidase from immature seeds of *G. max* and from *A. officinalis* shoots was partially purified using the methods of Fujii and Izawa [14] (Table 2). The specificities of γ-glutamyltranspeptidases in *G. max*, *P. vulgaris* [2] and *A. officinalis* were very similar (Table 3) and did not reflect the difference in γ-glutamyl peptide pattern between mature seeds of *G. max* and *P. vulgaris* and shoots of *A. officinalis*. Immature seeds of *G. max* and *A. officinalis* shoots

Table 1. γ-Glutamyltranspeptidase activity and contents of γ-glutamyl peptides in some leguminous plants and in *Asparagus officinalis*

| Plant species | Immature seeds | | | Mature seeds | | Seedlings or shoots | |
|---|---|---|---|---|---|---|---|
| | γ-Glutamyl-transpeptidase activity* pH 8.0 | pH 9.6 | Contents of γ-glutamyl peptides | γ-Glutamyl-transpeptidase activity* | Contents of γ-glutamyl peptides | γ-Glutamyl-transpeptidase activity* pH 8.0 | Contents of γ-glutamyl peptides |
| *Phaseolus radiatus* | 0.00 | 0.00 | | | γ-Glutamylmethionine and γ-glutamylmethionine sulfoxide in high concentrations [6–8] (cf. Table 5), γ-glutamylleucine [18], γ-glutamyl-γ-glutamylmethionine [21]. Traces of γ-glutamyltyrosine and γ-glutamylphenylalanine [18]. | | Low concentration of γ-glutamylmethionine. Traces of γ-glutamylmethionine sulfoxide (cf. Table 5) |
| *Glycine max* | 0.06 | 0.14 | None present | | γ-Glutamylphenylalanine and γ-glutamyltyrosine in high concentrations [9, 10] | High (at pH 9.6) [5] | Low concentrations of γ-glutamylaspartic acid [22] |
| *Phaseolus angularis* | † | 0.04 | Traces of γ-glutamyl-β-phenyl-β-alanine | | γ-Glutamyl-β-phenyl-β-alanine in high concentration [11] | | |
| *Vicia faba* | 0.00 | 0.03 | None present | | Traces of γ-glutamylphenylalanine, γ-glutamyltyosine and γ-glutamylaspartic acid [12] | | |

Table 1—*continued*

| Plant species | Immature seeds: γ-Glutamyl-transpeptidase activity* pH 8.0 | Immature seeds: γ-Glutamyl-transpeptidase activity* pH 9.6 | Immature seeds: Contents of γ-glutamyl peptides | Mature seeds: γ-Glutamyl-transpeptidase activity* | Mature seeds: Contents of γ-glutamyl peptides | Seedlings or shoots: γ-Glutamyl-transpeptidase activity* pH 8.0 | Seedlings or shoots: Contents of γ-glutamyl peptides |
|---|---|---|---|---|---|---|---|
| *Pisum sativum* | −0.02 | 0.00 | None present | 0.02 [2]‡ | None present | | γ-Glutamylhomoserine [23], γ-glutamyl-D-alanine [24] |
| *Phaseolus vulgaris* | | 0.01 [2]‡ | | Very low activity [2]‡ | γ-Glutamyl-*S*-methylcysteine and γ-glutamyl-*S*-methylcysteine sulfoxide [25], γ-glutamylleucine and γ-glutamylmethionine [26] | | |
| *Asparagus officinalis* | | | | | | 1.89 | Traces of γ-glutamylglutamic acid, γ-glutamylaspartic acid and γ-glutamyltyrosine [13] |

See text for incubation conditions.
*μmol *p*-nitroaniline formed/g fr. wt/hr.
†Not determined.
‡γ-Glutamylanilide was used instead of γ-glutamyl-*p*-nitroanilide at pH 9.0.

Table 2. Partial purification of γ-glutamyltranspeptidase from shoots of *Asparagus officinalis* (*A.o.*) and from immature seeds of *Glycine max* (*G.m.*)

| | Volume (ml) | | *p*-Nitroaniline released* (μmol/ml/hr) | | Protein (mg/ml) | | Specific activity | |
|---|---|---|---|---|---|---|---|---|
| | *A.o.* | *G.m.* | *A.o.* | *G.m.* | *A.o.* | *G.m.* | *A.o.* | *G.m.* |
| 1. Crude extract | 160 | 220 | 0.24 | 0.14 | 4.8 | 70.4 | 0.05 | 0.002 |
| 2. $(NH_4)_2SO_4$, 30% supernatant | 175 | 230 | 0.19 | 0.14 | 2.1 | 36.2 | 0.09 | 0.004 |
| 3. $(NH_4)_2SO_4$, 30–70% ppt. dialysed against tap water and centrifuged | 18 | 70 | 3.2 | 0.67 | 16.7 | 58.7 | 0.21 | 0.012 |
| 4. Dialysed against buffer 1† and centrifuged | 22 | 77 | 2.5 | 0.80 | 11.8 | 44.3 | 0.22 | 0.018 |
| 5. Acetone 1.5 vol. ppt. dialysed against tap water and buffer 1† and centrifuged | 13 | 29 | 1.9 | 1.03 | 4.3 | 29.8 | 0.44 | 0.035 |

*A. officinalis* (19 g) was homogenized with buffer 2† (150 ml) to obtain crude extract. Immature seeds of *G. max* were homogenized with buffer 2† (1 : 1, v/w) to obtain crude extract. See text for incubation conditions.

*The enzyme from *A. officinalis* was assayed in buffer 3† and the enzyme from *G. max* was assayed in buffer 4†.

†See text for compositions of buffer solutions.

Table 3. Specifities of γ-glutamyltranspeptidase from immature seeds of *Glycine max* and *Phaseolus vulgaris* and shoots of *Asparagus officinalis*

| Glutamyl acceptors | Relative activity | | | |
|---|---|---|---|---|
| | Immature seeds of | | Shoots of | |
| | *G. max* | *P. vulgaris* [2] | *A. officinalis* | [14] |
| *S*-Methyl-L-cysteine | 107 | 100 | 110 | * |
| L-Aspartic acid | 6 | 2 | 2 | −13 |
| L-Threonine | 5 | * | 14 | 8 |
| L-Serine | 7 | * | 28 | 36 |
| L-Glutamic acid | 12 | 17 | 0 | −20 |
| Glycine | 4 | 20 | 12 | 17 |
| L-Alanine | 1 | 0 | 20 | 25 |
| L-Valine | −1 | 53 | 8 | 10 |
| L-Methionine | 100 | 148 | 100 | 100 |
| L-Leucine | 44 | 84 | 75 | 77 |
| L-Tyrosine | 49 | 57 | 35 | 2 |
| L-Phenylalanine | 57 | 92 | 91 | 103 |
| L-Arginine | 52 | * | 47 | 62 |
| L-Asparagine | 23 | 43 | 39 | 51 |
| L-Glutamine | 29 | 30 | 56 | 3 |

See text for incubation conditions (buffer 4 for *G. max* and *P. vulgaris*, buffer 3 for *A. officinalis*).

*Not determined.

contain much higher concentrations of asparagine and glutamine (nearly all of the amide-N is asparagine in *G. max*) than of other free amino acids (Table 5). Asparagine and glutamine are relatively good acceptors in γ-glutamyl transpeptidation (Table 3), but neither γ-glutamylasparagine nor γ-glutamylglutamine has been found in mature seeds of *G. max* or in *A. officinalis* shoots.

Although the γ-glutamyltranspeptidase activity of immature seeds of *P. radiatus* was very low, the activity increased during germination, accompanied by a decrease of γ-glutamylmethionine and its sulfoxide in cotyledons (Tables 4 and 5). Goore and Thompson have reported that a purified enzyme of *P. vulgaris* had no transpeptidase activity below pH 7.5, whereas the hydrolytic reaction showed optimum activity at both 9.5 and 6.5 [3]. No γ-glutamyltranspeptidase activity was detected in dormant bulbs of *Allium cepa* [2], but the activity was present in sprouting bulbs [15].

Table 4. Changes in γ-glutamyltranspeptidase activity during germination of *Phaseolus radiatus*

| | g fr.wt/10 samples | μmol *p*-nitroaniline released in buffer 4/plant/hr |
|---|---|---|
| Seedlings 1* | | |
| Cotyledons | 1.2 | 0.04 |
| Hypocotyls, radicles | 0.2 | 0.01 |
| Seedlings 2* | | |
| Cotyledons | 1.0 | 0.03 |
| Hypocotyls, radicles | 0.7 | 0.03 |
| Seedlings 3* | | |
| Cotyledons | 1.0 | 0.05 |
| Hypocotyls, radicles | 0.8 | 0.03 |
| Seedlings 4* | | |
| Cotyledons | 1.0 | 0.09 |
| Hypocotyls, radicles | 1.4 | 0.03 |
| Seedlings 5* | | |
| Cotyledons | 0.8 | 0.14 |
| Hypocotyls, radicles | 1.6 | 0.04 |
| Seedlings 6* | | |
| Cotyledons | 0.3 | 0.14 |
| Hypocotyls, radicles | 3.0 | 0.07 |

See text for incubation conditions.

*The length of hypocotyl plus radicle of seedlings 1 to 6 are 1–1.5 cm, 3–4 cm, 5–6 cm, 8–10 cm, 11–13 cm and 18–20 cm, respectively.

The major γ-glutamyl peptide in shoots of *A. officinalis*, although in very low concentration, is γ-glutamylglutamic acid, in conflict with the fact that glutamic acid is a very poor acceptor for γ-glutamyltranspeptidase in *A. officinalis* (Table 3) [13]. During glutamic acid fermentation of *Corynebacterium glutamicum*, γ-glutamylglutamic acid is formed in the broths directly from glutamic acid by the reversal of hydrolysis catalysed by γ-glutamyltranspeptidase [16]. Glutamic acid solution of the same concentration as that of glutamic acid in *A. officinalis* shoots was incubated with γ-glutamyltranspeptidase partially purified from *A. officinalis* to see whether the mechanism of γ-glutamylglutamic acid formation observed in glutamic acid fermentation also operates in *A. officinalis* shoots. No γ-glutamylglutamic acid was produced in the incubation mixture. The results reported here indicate that γ-glutamyltranspeptidases play a role in degradation rather than in biosynthesis of γ-glutamyl peptides. Transpeptidase activity is high when net degradation of γ-glutamyl peptides takes place, but low when net production takes place. Furthermore, the contents of γ-glutamyl peptides are not correlated with the contents of free amino acids. In some cases the γ-glutamyl peptides cannot be produced by the transpeptidase because of the low specificity of the enzyme for the corresponding amino acids. Finally, the transpeptidases act mainly as peptidases at the pH values present in the plants [3]. However, if γ-glutamyl peptides are not produced by transpeptidase action, then no other proposal is presently available for their production. γ-Glutamylcysteine synthetase with low specificity may play a role.

Transpeptidation activity of the enzyme prepared from *A. officinalis* was confirmed by isolation of γ-glutamylmethionine from the incubation mixture of γ-glutamyl-*p*-nitroanilide, methionine and enzyme solution.

## EXPERIMENTAL

*General method.* Extraction and incubation were performed with the following buffers: 0.01 M acetate buffer (pH 5) (buffer 1); 1% NaCl in buffer 1 (buffer 2), 1 M Na citrate in 0.5 M Tris buffer (pH 8.0 and 9.6 with HOAc) (buffers 3 and 4, respectively). HVE was performed at pH 3.6 (pyridine–HOAc–$H_2O$, 1:20:200, 55 V/cm) (buffer 5) and pH 6.5 (pyridine–HOAc–$H_2O$, 25 : 1 : 500, 100 V/cm) (buffer 6). 2D-PC was carried out with *n*-BuOH–HOAc–$H_2O$ (4 : 1 : 2) (solvent 1) and PhOH–$H_2O$–18 M $NH_4OH$ (120:30:1, w/v/v) (solvent 2).

*Plant materials.* Immature seeds of *P. radiatus*, *P. angularis*, *V. faba* and *P. sativum* were harvested at the experimental farm of Hokkaido University. Immature seeds of *G. max* and green shoots of *A. officinalis* were purchased at the market. Seedlings of *P. radiatus* were grown as follows: seeds of *P. radiatus* were soaked in running $H_2O$ overnight and germinated at 20° in the dark for several days. Seedlings of nearly the same size were harvested at intervals.

*Determination of protein.* Protein was determined by the Na carbonate Folin method [17].

Table 5. Contents of major amino acids and amino acids which are also present as γ-glutamyl derivatives in seeds of *Glycine max*, shoots of *Asparagus officinalis* and seeds and seedlings of *Phaseolus radiatus*

| | *G. max* Seeds | | *A. officinalis* shoots | *P. radiatus* | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Seeds | | Seedlings | | | |
| | | | | | | Cotyledons | | Hypocotyls ·radicles | |
| | Immature (μmol/10 seeds) | Mature [20] (μmol/10 seeds) | (μmol/g fr. wt) | Immature (μmol/10 seeds) | Mature [7] (μmol/10 seeds) | 2* | 3* | 2* | 3* |
| | | | | | | (μmol/10 samples) | | | |
| Aspartic acid | 9.5 | 6.3 | 1.0 | 1.9 | 0.8 | 3.2 | 3.0 | 0.3 | 2.9 |
| Asparagine | ++† | +† | +++† | +† | ++† | ++† | ++† | ++† | ++++† |
| Glutamine | ±† | +† | +++† | +† | | +† | +† | +† | ±† |
| Glutamic acid | 17.0 | 7.4 | 2.2 | 3.8 | 1.0 | 8.1 | 2.4 | 0.2 | 1.3 |
| Proline | | ± | 2.5 | ± | 0.4 | | | 0.7 | |
| Alanine | 13.3 | 1.4 | 2.4 | 4.8 | 0.3 | 1.5 | 1.0 | 1.6 | 3.3 |
| Methionine | 0.5 | | | 0.3 | 0.8 | 0.3 | 0.3 | 0.3 | 0.6 |
| Leucine | 1.1 | 0.3 | ± | 0.4 | 0.1 | 0.5 | 0.7 | 0.6 | 1.9 |
| Tyrosine | 0.5 | 0.7 | | 0.5 | ± | ± | ± | ± | ± |
| Phenylalanine | 1.3 | 0.8 | ± | 0.3 | 0.1 | ± | 1.1 | 0.6 | 2.5 |
| Arginine | 10.7 | 16.0 | ± | 1.6 | 0.3 | | | ++ | 2.8 |
| Amide-$NH_3$ | 37.7 | ‡ | 16.4 | ‡ | 4.3 | ‡ | ‡ | ‡ | ‡ |
| γ-Glutamyltyrosine | | 3.4 | ± | | ± | | | | |
| γ-Glutamylphenylalanine | | 3.4 | | | ± | | | | |
| γ-Glutamylmethionine | | | | | 2.6 | 2.4 | 1.1 | | |
| γ-Glutamylmethionine sulfoxide | | | | | +++ | ± | ± | | |
| γ-Glutamylleucine | | | | | + | ± | | | |

*See footnote of Table 4 for seedlings 2 and 3.

†The peaks for asparagine and glutamine overlapped on an amino acid analyser.

‡Not determined.

The amount of amino acids and peptides which could not be separated by an amino acid analyser was estimated by 2D-PC. Relative concentrations are indicated by ++++ to ±.

*Substrate.* DL-γ-Glutamyl-*p*-nitroanilide soln (4.5 mM) was used as the substrate for the γ-glutamyltranspeptidation reaction.

*Assay for γ-glutamyltranspeptidase activity.* Plant materials were homogenized with an equal amount (1 g/1 ml) of buffer 2 or 0.01 M $NaHCO_3$ and centrifuged. The supernatant was used for the analysis of transpeptidase activity. The composition of incubation mixtures, incubation conditions and assay procedures were as follows: enzyme soln (0.5 ml), substrate soln (0.2 ml), 0.05 M *S*-methyl-L-cysteine (0.2 ml) as acceptor of the γ-glutamyl residue, buffer 3 or 4 (0.5 ml), 37°, 2 hr, stop with 1.5 M HOAc (2.6 ml), centrifugation and measurement of OD at 410 nm. Activities of γ-glutamyltranspeptidase were expressed by the difference between the amount of *p*-nitroaniline liberated in the presence and in the absence of *S*-methyl-L-cysteine in the incubation mixtures.

*Partial purification of γ-glutamyltranspeptidase from immature* G. max *seeds and* A. officinalis *shoots and their specificities.* γ-Glutamyltranspeptidase in immature seeds of *G. max* and *A. officinalis* shoots were partially purified by the method of ref. [14]. Specificities of the partially purified enzymes were determined with various amino acids as acceptors instead of *S*-methyl-L-cysteine. Glutamic acid (final concn, 2.2 μmol/ml, corresponding to the concn of glutamic acid in *A. officinalis* shoots (Table 5) was incubated with γ-glutamyltranspeptidase prepared from *A. officinalis* in buffer 3 at 37° for 2 hr. No ninhydrin-positive substance except glutamic acid was detected in the incubation mixture by the use of an amino acid analyser.

*Contents of free amino acids and γ-glutamyl peptides.* Immature seeds of *G. max* and *A. officinalis* shoots were homogenized in 10 vol. 70% EtOH. The filtrate was concd and applied to a column of Dowex 50($H^+$), which was thoroughly washed with $H_2O$. The amino acid fraction was eluted from the column with 2 M $NH_4OH$ and analysed by 2D–PC, HVE and an amino acid analyser. Seedlings of *P. radiatus* were separated into cotyledons and remaining parts (hypocotyls and radicles). Amino acid fractions from the separate tissues obtained by the procedures described above were applied to a column of Dowex 1 × 4($AcO^-$). The fraction of basic and neutral amino acids was washed out with $H_2O$ and the fraction of acidic amino acids was eluted with 2 M HOAc. Each fraction was analysed by 2D–PC, HVE and the amino acid analyser. Amide-$NH_3$ was determined by hydrolysis (1 M HCl, 105°, 2 hr in sealed tube) and $NH_3$ determination.

*Isolation of γ-glutamylmethionine from the incubation mixture.* γ-Glutamyl-*p*-nitroanilide (10 mg) and methionine (60 mg) were incubated with the partially purified enzyme soln (5 ml) from *A. officinalis* shoots in buffer 3 (total vol. 40 ml, 37°, 5 hr). The incubation mixture was applied to a column of Amberlite IR-120 ($H^+$, 50 ml), which was thoroughly washed with $H_2O$. The fraction of amino acids obtained by elution with 2 M $NH_4OH$ from the column was applied to a Dowex 1 × 4 column ($OH^-$, 2 ml). After washing with $H_2O$, the amino acid fraction was obtained by elution with 2 M HOAc. Fractions (2 ml each) were collected. Fractions 1–6 were concd and applied to a Dowex 1 × 4 column ($AcO^-$, 2 ml), which was washed with $H_2O$ and eluted with 0.2 M HOAc (20 ml) and 2 M HOAc (8 ml). γ-Glutamylmethionine was isolated from the 2 M HOAc eluate by purification in prep. HVE with buffer 6. FDMS (*m/z*): 279 ($M^+ + 1$, 100%). The chromatographic behaviour of the isolated material on 2D–PC, HVE and the amino acid analyser was identical to that of authentic γ-glutamylmethionine isolated from *P. radiatus* seeds [18, 19]. The yield was very low (0.7 mg). The isolated peptide gave two peaks for glutamic acid and methionine (1 : 1 molar ratio) on an amino acid analyser after hydrolysis (1 M HCl, 105°, 3 hr).

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