The Isolation and Characterization of γ-L-Glutamyl-L-Tyrosine and γ-L-Glutamyl-L-Phenylalanine from Soybeans

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Two dipeptides of glutamic acid have been found in the nonprotein nitrogen fraction of soybeans. They have been isolated by extraction with ethanol-water, chromatography on ion-exchange resins, and recrystallization from ethanol-water. Confirmation of structure has been established by degradation and quantitative estimation of the products, and by synthesis and comparison of the isolated and synthetic samples. It was thus possible to prove that the two dipeptides were γ-L-glutamyl-L-tyrosine and γ-L-glutamyl-L-phenylalanine.

Prior to 1958 γ-glutamyl dipeptides had not been isolated in crystalline form from plants. In that year two papers were published on the isolation of γ-L-glutamyl-*S*-methyl-L-cysteine from kidney beans (Zacharius *et al.*, 1958; Rinderknecht *et al.*, 1958). Primarily through efforts at this laboratory and at Virtanen's, there have since been reports of the isolation of eight other γ-glutamyl dipeptides from higher plants (Morris *et al.*, 1961; Thompson *et al.*, in press; Virtanen and Matikkala, 1961). This paper records the isolation and characterization of γ-L-glutamyl-L-tyrosine and γ-L-glutamyl-L-phenylalanine from soybeans.

Evidence for the Natural Occurrence of γ-Glutamyl Tyrosine and γ-Glutamyl Phenylalanine

Paper chromatographic examination of the acidic acids in the nonprotein fractions of soybean seeds revealed four ninhydrin-reactive materials which occupied unusual positions on the chromatogram. Two of the compounds were present in amounts that appeared comparable to those of uncombined aspartic and glutamic acids. After it was discovered that these two compounds were labile to acid hydrolysis, small amounts of each were isolated by paper chromatography. Hydrolysis of each of the isolated compounds indicated that one contained glutamic acid and tyrosine while the other was made up of glutamic acid and phenylalanine.

Several hundred milligrams of the two compounds were isolated in crystalline form by chromatography on the acetate form of Dowex 1. Elemental analysis and quantitative determination of the products of hydrolysis showed that two amino acids accounted for all of the weight in each compound and that the two acids were in a one-to-one ratio. Quantitative reaction of the compounds with ninhydrin strongly suggested that these were dipeptides.

Evidence that each peptide contained a free amino group adjacent to an uncombined carboxyl made it appear likely that these were γ-glutamyl dipeptides. γ-L-Glutamyl-L-tyrosine and γ-L-glutamyl-L-phenylalanine were synthesized and found to be identical to the isolated peptides in infrared spectra, optical rotation, elemental analysis, and chromatography on paper and ion-exchange resins.

Experimental

Preliminary Identification.—Dry soybean seeds were powdered in a micropulverizer. Fifty grams of powder were thoroughly extracted at room temperature by repeated treatment with 80% ethanol. Lipids were removed by the addition of chloroform and water to the alcohol extract. Total amino content was determined by ninhydrin after removal of ammonia (Connell *et al.*, 1955).

An aliquot containing 1 mg of amino nitrogen was treated with ion-exchange resins to purify the amino acids (Thompson *et al.*, 1959).

Since the discovery of several γ-glutamyl peptides and unusual acidic amino acids (Morris *et al.*, 1959; Thompson *et al.*, 1961) in plants, routine procedure in this laboratory has been to separate the acidic amino acid fraction from the neutral and basic amino acids. In order to accomplish this, Dowex 1-×4 (100–200 mesh) was activated in a column by treatment successively with 10 column volumes of 1 N NaOH, 10 column volumes of water, 5 column volumes of 2 N acetic acid, and deionized water until the effluent was neutral.

The neutral and acidic amino acid fraction was put on a column of the acetate resin (7.0 × 0.9 cm) and the neutral amino acids were washed through with deionized water at 5° until the effluent no longer gave a positive ninhydrin test on paper. The acidic amino acids were eluted with 80 ml of cold 2 N acetic acid and the solvent immediately removed in a rotary evaporator at 35°. The residue was dissolved in 10 ml of water and a 1-ml aliquot was taken for two-directional paper chromatography (Thompson and Morris, 1959) in *n*-butanol–acetic acid–water (9:1:2.5) and phenol–water (8:3). Development of the chromatograms with ninhydrin revealed four unusual spots, two of which were very strong and com-

parable in density to aspartic and glutamic acids. Small quantities of the two prominent unknown compounds were isolated by one-directional paper chromatography and found to be unstable to acid hydrolysis (3 hours in 3 N HCl at 120°). The products of one of the compounds co-chromatographed with glutamic acid and tyrosine, whereas those of the other compound coincided with glutamic acid and phenylalanine.

The results indicated that the unknown compounds were peptides of glutamic acid and tyrosine and of glutamic acid and phenylalanine.

Isolation.—Yellow soybeans (25 kg) were powdered and thoroughly extracted at room temperature with 70% ethanol. The extracts were cooled to 5° and, after remaining at this temperature for several days, the clear supernatants were put through a Dowex 50 column (hydrogen form, 5°). Because the beans were not defatted before extraction, there was considerable precipitation of material on the resin columns. This material was not removed by the alcohol or water wash but was redissolved during elution with 2 N ammonia. The eluate was evaporated at 40° *in vacuo*, the residue dissolved in water, and the contaminant removed by precipitation at *p*H 4.0.

The partially purified amino acids were absorbed on a 5.8 × 127 cm column of Dowex 1 Ac (200–400 mesh) and washed thoroughly with deionized water to remove neutral and basic amino acids. The initial eluant was 0.1 N acetic acid, and 21-ml fractions were collected at a flow rate of 3.5 ml per minute. The normality of the acetic acid was changed to 0.3 at fraction 900, and to 1.0 at fraction 1400, and 2.0 N acetic acid was introduced to the column at fraction 2400. One drop of solution from alternate fractions was placed, in rows, on a large sheet of filter paper, dried, and sprayed with a 0.5% solution of ninhydrin in ethanol. The density of the color indicated the tubes containing the peak amino acid concentrations. The peaks were then investigated by using small (18 × 18 cm) two-directional chromatograms, which indicated that fractions 2000–2300 contained the glutamic-phenylalanine peptide and fractions 2630–2870 contained the glutamic-tyrosine peptide. Fractions 2000–2300 were combined, as were 2630–2870, the solvent was removed *in vacuo*, and the compounds were crystallized from water as colorless solids. After several recrystallizations, several hundred milligrams of each peptide were obtained as colorless crystals. For elemental analysis, optical rotation, and other information, see Table II.

Proof of Structure.—Twenty mg of each of the isolated compounds was heated for 4 hours with 3 N HCl at 120° and the acid removed. The residues were dissolved in water and an aliquot taken for analysis with the amino acid analyzer using the buffered resin system (Moore *et al.*, 1958). The analysis revealed a nearly 1:1 ratio of glutamic acid to the other amino residue (see Table I). Glutamic acid and tyrosine in the one peptide, and glutamic acid and phenylalanine in the other, accounted for essentially all of the weight. The identity of the hydrolytic products was further confirmed by the fact that their elution volumes corresponded exactly with those of the standard amino acids.

TABLE I

PERCENTAGE OF RECOVERY OF AMINO ACIDS FROM PEPTIDES[a]

	γ-Glutamyl Phenylalanine		γ-Glutamyl Tyrosine	
	Isolated	Synthetic	Isolated	Synthetic
Phenylalanine	94	95	--	--
Glutamic acid	96	100	102	102
Tyrosine	--	--	98	99

[a] Comparison of 1 μmole of standard glutamic acid, tyrosine, and phenylalanine with an aliquot of hydrolyzed peptide calculated to contain 1 μmole of each amino acid, assuming a dipeptide. Data obtained with an amino acid analyzer and the buffered resin system of Moore *et al.* (1958).

Quantitative reaction of each of the peptides with ninhydrin (Connell *et al.*, 1955) in a test tube indicated that they were both dipeptides. There was the possibility of the peptide bond breaking during the ninhydrin reaction, thus liberating another amino group and giving an erroneous impression of the molecular weight. This was checked by varying the heating time from 5 minutes to 1 hour with no appreciable change in ninhydrin values.

A few milligrams of the two original compounds were reacted with fluorodinitrobenzene according to Levy and Chung (1955) and the products hydrolyzed. After removal of the acid, a water solution of each material was put through a short column of Dowex 50 in the hydrogen form, and thoroughly washed with water. The amino acid moiety, which was atached to the dinitrophenyl group, remained in the effluent wash, while the free amino acid was absorbed on the resin. Elution of the two columns with ammonia and chromatography of the products showed that in both compounds glutamic acid contained the free amino group.

A solution of each of the two peptides containing 2 mg per ml was spotted on filter paper and reacted with an alcoholic solution of pyridoxal followed by a ninhydrin spray (Kalyankar and Snell, 1957). This test indicated that there was a free amino group adjacent to a free carboxyl in each isolate, and the only possible configuration that would satisfy this requirement would be a γ-glutamyl peptide. The two dipeptides were then synthesized in order to make critical comparisons.

Synthesis and Comparison.—Tri-*n*-butylamine was added to *N*-carbobenzyloxy-α-benzyl-L-glutamic acid (Cyclo Chemical Company, Los Angeles, Calif.) in 10 ml of dioxane. The mixture

TABLE II
INFORMATION ON ISOLATED AND SYNTHETIC DIPEPTIDES

	γ-Glutamyl Tyrosine		γ-Glutamyl Phenylalanine	
	Isolated	Synthetic	Isolated	Synthetic
Elemental Analysis				
% Obtained				
Carbon	54.6	54.3	56.6	56.8
Hydrogen	5.88	6.21	6.42	6.35
Nitrogen	9.08	9.01	9.75	9.21
% Theory				
Carbon	54.3	—	57.1	—
Hydrogen	5.85	—	6.12	—
Nitrogen	9.03	—	9.53	—
$[\alpha]_D^{31}$ C = 4	+26.6°	+25.5°	+16.9°	+16.9°
R_F Values				
Butanol-acetic acid-water (9:1:2.5)	0.35	0.35	0.55	0.55
Phenol-water (8:3)	0.57	0.57	0.75	0.75
Melting points	221–222°d	219–221°d	164–174°d	164–174°d

was cooled to 5° and ethyl chloroformate added (Sachs and Brand, 1953). In each instance 0.005 mole of reactant was used. This was allowed to remain for 30 minutes at 5°, and 12.5 ml of cold dioxane containing 0.0065 mole of L-tyrosine benzyl ester, *p*-toluene sulfonic acid salt (Cyclo Chemical Co.), and 0.0065 mole of tri-*n*-butylamine was added. The reaction mixture remained in the cold overnight, and to it was added 50 ml of ethyl acetate followed by 75 ml of 0.5 N HCl. After shaking the acetate layer was separated and washed with 5% $NaHCO_3$ followed by H_2O. The solvent was removed with a rotary evaporator and the solid crystallized from aqueous ethanol. The free peptide was obtained by catalytic hydrogenation for 6 hours with 1 g of palladium at atmospheric pressure in 100 ml of 90% acetic acid. The peptide was absorbed on a short column of Dowex 1 in the acetate form and thoroughly washed with 0.1 N acetic acid. After elution with 2 N acetic acid, the peptide was recrystallized several times from ethanol-water to give 500 mg of white crystals.

Synthetic γ-L-glutamyl-L-phenylalanine was prepared in a manner exactly analogous to the above synthesis except that L-phenylalanine benzyl ester, *p*-toluene sulfonic acid salt (Cyclo Chemical Co.) was used instead of the tyrosine ester. The yield of synthetic peptide was approximately 33% of the theoretical amount.

Comparisons of the synthetic and isolated compounds are shown in Table II. Paper chromatography of the compounds in two solvent systems failed to separate the synthetic compound from the isolated one in either case. The infrared absorption spectra of KBr disks of the synthetic and isolated materials were indistinguishable. The optical rotations of the peptides in water were very similar to those of the isolated compounds (Table II).

DISCUSSION

Since a considerable number of γ-glutamyl dipeptides have recently been discovered in plants, it was of interest to determine the relative quantities of the two dipeptides in soybeans and to learn something of their formation. The content of dipeptides and related compounds is presented in Table III. The data demonstrate that each dipeptide contributes an appreciable portion of the nonprotein nitrogen, and that the amounts of tyrosine and phenylalanine in the peptide form are considerably greater than in the free form. These observations are similar to those made on the kidney bean with respect to γ-glutamyl methyl cysteine and to γ-glutamyl-β-aminoisobutyric acid in the iris bulb (Thompson *et al.*, in press).

Virtanen and Matikkala (1961) have recently reported the isolation of γ-glutamyl phenylalanine from onions. Since onions are taxonomically unrelated to beans, a wide distribution of γ-glutamyl phenylalanine among higher plants may be possible. This is the first published report of the natural occurrence of γ-glutamyl tyrosine.

Preliminary evidence has indicated that these

TABLE III
CONTENT OF AMINO COMPOUNDS IN NONPROTEIN FRACTION OF SOYBEAN SEEDS

	Amount		
Compound	μg/g Fresh Wt.	μmoles 100 μatoms Nonprotein Nitrogen	Mole Ratio of Peptide to Free Form
Glutamic acid	636	17.3	0.55
Tyrosine	16	0.18	27.5
Phenylalanine	57	0.70	6.7
γ-Glutamyl-tyrosine	384	4.9	
γ-Glutamyl-phenylalanine	393	4.7	

Values obtained by quantitative paper chromatography (Thompson and Morris, 1959).

γ-glutamyl dipeptides are formed from glutathione and an amino acid in the presence of a γ-glutamyl transpeptidase (Thompson *et al.*, in press).

The lengthening list of amino substances which are found in the form of γ-glutamyl compounds in diverse places in the plant kindgom suggests that they may play an interesting role in the nitrogen metabolism of plants.

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Isolation Studies of Allergens from Ragweed Pollen*

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A highly allergenic protein preparation apparently of 32,000 molecular weight has been isolated from the ragweed pollen extract by ammonium sulfate precipitation followed by chromatography on DEAE cellulose and on Sephadex. A second component of about 10,000 molecular weight was found to be also highly reactive in four out of thirty-two untreated hay fever patients. A cause for the gradual loss of the allergenic activity of ragweed pollen extract on storage is suggested.

One of the most common seasonal hay fevers is caused by ragweed pollen. Extensive studies designed for the isolation and characterization of the active allergens have been under way in many different laboratories ever since the recognition that this pollen is the causative agent. These studies have been reviewed by several different authors (Newell, 1941; Sherman, 1959; Richter and Sehon, 1960).

In spite of all this careful work, the objective has not thus far been realized and the real chemical nature of the allergen is not known. The results from most laboratories indicate the activity to be associated with protein fractions, but others indicate it to be present in peptide fractions. Obviously the problem is one of considerable difficulty and one which presents a challenge to any laboratory interested in the isolation and characterization of active principles. It has been taken up with the hope that certain techniques being developed in this laboratory may prove of value.

One major difficulty in the isolation of the ragweed allergens is the lack of methods capable of providing quantitative estimations of the allergenic activity. At present estimations are usually made by direct skin tests on sensitized individuals or by indirect skin tests on normal individuals passively sensitized with sera from ragweed patients (Sherman, 1959). Both tests are subject to at least tenfold variations. Because of such variations, it is helpful to have an independent *in vitro* assay method. One such possible method is agar gel diffusion analysis of antigens with precipitating antibodies from rabbits immunized with ragweed pollen extracts. In the present work, the Ouchterlony analysis of antigens was used for rapid identification and comparison of different ragweed antigens. Since the substances which are antigenic in rabbits are not necessarily the allergens in man, direct skin tests were carried out later on the isolated fractions to identify the allergens.

This paper will describe the isolation of a highly allergenic protein preparation of 32,000 molecular

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