Synthesis and Characterization of a Glutamic Acid Enriched Plastein with Greater Solubility

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A 2:1 mixture of a peptic hydrolysate of soybean protein and L-glutamic acid α,γ-diethyl ester was incubated with papain. A plastein was thus synthesized in a yield of 95.7 g from 100 g of hydrolysate. This plastein contained 41.93% glutamic acid residue and was almost completely soluble in water at pH 1–9. No insoluble fraction was formed from the plastein solution during heating at 100°. Other physicochemical properties specific to this plastein were as follows: average molecular weight, 6240; isoelectric point distribution, pH 1.5–4.0; and circular dichroism, a spectral trough at 222 nm (assignable to the α-helix structure). Data were added concerning *in vitro* digestibilities and tastes arising after the digestion.

A nutritional application of the plastein reaction has been reported in the example of enhancing a methionine level of soybean protein (Yamashita *et al.*, 1971; Arai *et al.*, 1974). Location and states of methionine incorporated to plastein molecules have been investigated in detail (Yamashita *et al.*, 1972). A similar technique has been applied to zein, and its tryptophan, threonine, and lysine levels have more or less been improved (Aso *et al.*, 1974a). However, a property common to most plasteins is their low water solubility which may sometimes be unfavorable in the process of making protein-enriched drinks, soups, confections, etc.

In considering that a highly water-soluble property is generally derived from a high content of some hydrophilic amino acid, we attempted preparing a plastein with an extremely high amount of glutamic acid. The present paper deals with a procedure for synthesizing such a plastein and with its water solubility and related physicochemical properties.

MATERIALS AND METHODS

Protein Hydrolysate. A soybean globulin preparation (N × 6.25 = 92%) was hydrolyzed with pepsin at pH 1.6 according to a previous paper (Yamashita *et al.*, 1971). The hydrolysate suspension (1% in concentration) was adjusted to pH 4.5 with NaOH and an insoluble fraction removed by centrifuging at 3000 rpm for 15 min. A soluble fraction was thus obtained in a yield of 85 g from 100 g of soybean globulin on a dry-matter basis. This fraction was freeze-dried.

L-Glutamic Acid Ethyl Esters. L-Glutamic acid α,γ-diethyl ester (Glu-α,γ-OEt_2) was prepared by the method of Boissonnas *et al.* (1956), L-glutamic acid α-ethyl ester (Glu-α-OEt) by the method of Le Quesne and Young (1950), and L-glutamic acid γ-ethyl ester (Glu-γ-OEt) by the method of Bergmann and Zervas (1933).

Substrate. A 2:1 (w/w) mixture of the peptic hydrolysate (soluble fraction) and each glutamic acid ester was used as a substrate for the plastein synthesis.

Plastein Synthesis. The following conditions were used: substrate concentration, 52.5% (w/v); solvent, 20% (v/v) acetone in 0.01 *M* L-cysteine (pH 5.5); enzyme, papain (Difco Laboratories, NF VIII); enzyme-substrate ratio, 1:50 (w/w); incubation temperature, 37°; and incubation time, 24 hr. After the incubation the entire reaction mixture was treated with a tenfold volume of 0.2 *N* NaOH for 2 hr at room temperature to cut off the remaining ethyl ester linkage and was dialyzed through a cellophane membrane against running water at 5° for 4 days to obtain a plastein as a nondialyzable fraction.

Plastein Yield Assay. Both the entire and the nondialyzable fractions were analyzed for nitrogen (Clark, 1943). The plastein yield was given on a hydrolysate basis and on a substrate basis (see Table I).

Amino Acid Analysis. A sample (1 mg) was added to 6 *N* HCl in a test tube. Air was exhaustively removed by a repeated freeze–thaw technique. The hydrolysis was carried out at 110° for 24 hr. The liberated amino acids were determined with a Hitachi amino acid analyzer (KLA-5). Half-cystine was analyzed according to Schram *et al.* (1954).

Solubility Test. A sample solution (suspension) was diluted to a concentration of 200 mg/100 ml and measured for the turbidity (absorbance) at 600 nm. The per cent solubility (x) was obtained from the observed absorbance (y) through the equation $y = 0.211(100 - x)$ (Yamashita *et al.*, 1970).

Estimation of Average Molecular Weight. A sample was analyzed for its total amino nitrogen content (Van Slyke, 1910), and the value corrected by the lysine-ϵ-amino nitrogen content. The average molecular weight was calculated from this corrected value on the assumption that a plastein molecule took the shape of a one-head, one-tail polypeptide.

Electrophoresis. A 7.5% polyacrylamide gel plate was prepared and equilibrated with phenol–acetic acid–water (1:1:1). Each sample was applied on this and electrophoresed at 3 mA/cm^2 for 7 hr. Staining was done with Amido Black 10B. A molecular weight *vs.* migration distance relation was obtained by the method reported by Tsai *et al.* (1974).

Isoelectric Focusing. Each sample (50 mg) was dissolved in 6 *M* urea (1 ml) and applied to the LKB Model 8101 (column capacity, 110 ml) which consisted of an Ampholite–6 *M* urea system with a sucrose density gradient. The sample was electrophoresed at 5° for 40 hr and every 1 ml was fractionated. Each fraction, after being measured for pH, was dialyzed through a cellophane membrane against running water for 3 days and the nondialyzable fraction freeze-dried. Each freeze-dried product was treated with 10% trichloroacetic acid and the precipitate determined by the turbidometry (Yamashita *et al.*, 1970).

In Vitro **Digestibility.** The following proteases were obtained from commercial sources: pepsin from Sigma Chemical Co., α-chymotryspin from Miles Laboratories, Molsin from Seishin Seiyaku Co., and Pronase from Kaken Kagaku Co. A plastein sample (1 g) was dissolved in water (100 ml) and the solution was adjusted to a given pH value with concentrated HCl or NaOH. An enzyme (20 mg) was added and the mixture incubated with vigorous shaking at 37° for 24 hr. After the incubation, the

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Table I. Yields of Plasteins and Their Glutamic Acid Contents

L-Glu and its esters	Yield, % Based on hydrolysate[a]	Yield, % Based on substrate[b]	Glu content, %[c]	Designation
L-Glu-α,-γ-OEt_2	95.7	63.8	41.93	Glu-plastein
L-Glu-α-OEt	54.5	36.3	32.18	
L-Glu-γ-OEt	58.1	38.7	25.34	
Free L-Glu	56.9	37.9	25.00	
None	60.0	60.0	24.81	Control plastein

[a] Peptic hydrolysate of soybean globulin. [b] A 2:1 mixture of this hydrolysate and L-glutamic acid or its ester. [c] See also Table II.

Table II. Amino Acid Composition of Plasteins and a Peptic Hydrolysate of Soybean Globulin

Amino acid	Hydrolysate, wt %	Glu-plastein,[a] wt %	Control plastein,[a] wt %
Ala	3.11	2.51	3.16
Arg	6.14	3.82	4.38
Asp	13.60	11.76	12.73
Glu	24.12	41.93[b]	24.81[b]
Gly	3.86	3.64	3.56
Half-cystine	1.75	1.60	1.80
His	2.11	1.22	1.60
Ile	5.60	2.37	5.08
Leu	7.14	3.65	7.32
Lys	4.22	3.05	3.30
Met	1.25	1.20	1.27
Phe	4.81	2.50	5.14
Pro	3.08	2.88	2.87
Ser	4.73	4.46	4.90
Thr	3.50	3.08	3.40
Tyr	1.59	0.88	2.31
Val	5.34	4.34	5.58

[a] For the designation see Table I. [b] See also Table I.

reaction mixture was treated with an equal volume of 20% trichloroacetic acid (TCA) and the resulting precipitate analyzed for nitrogen (Yamashita *et al.*, 1970). The digestibility was given as (10% TCA-soluble nitrogen/total nitrogen) × 100.

Taste Evaluation. Each plastein digest (1% in concentration) was neutralized to pH 6.0 with concentrated HCl or NaOH and evaluated for bitter and brothy tastes. A panel of six trained members was employed for the evaluation. A one-to-five rating scale as described in detail in Table V was used.

RESULTS AND DISCUSSION

A functional property important to a food protein material is its high solubility in water. Such a property depends partly on the amino acid composition of this material, especially on its hydrophilic amino acid content. For this reason we tried to prepare a protein-like substance (plastein) which contained a large amount of glutamic acid residue.

It is possible to alter an amino acid pattern of a given protein hydrolysate by incorporating amino acid esters

Table III. Solubilities[a] of Plasteins and a Soybean Protein in Water at Various pH Values

Sample[b]	pH 1	2	3	4	5	6	7	9
Glu-plastein	98.1	98.0	97.7	97.5	97.7	98.0	98.3	98.3
Control plastein	90.2	88.5	81.0	75.0	77.2	80.3	83.5	87.4
Soybean protein	75.1	80.2	70.3	12.5	18.3	44.4	83.7	97.5

[a] Each sample (1 g) was treated with water (100 ml) and its solubility given as (soluble nitrogen/total nitrogen) × 100. [b] For the designation see Table I.

Table IV. Solubility Changes by Heating[a]

Sample[b]	Heating time, min 0	5	10	15	30	60
Glu-plastein	97.0	96.2	95.8	95.6	95.8	95.6
Control plastein	33.5	32.9	32.2	31.8	31.0	31.1
Soybean protein	95.2	75.3	50.3	38.3	35.7	33.3

[a] Each sample (800 mg) was added to water (10 ml) and the solution (suspension) heated at 100°. After the heating, the water-soluble or insoluble fraction was gathered by centrifuging. The solubility was given as (soluble nitrogen/total nitrogen) × 100. [b] For the designation see Table I.

with the aid of the plastein reaction (Yamashita *et al.*, 1971). It has been reported that some hydrophobic amino acid esters are more efficiently incorporated during the plastein reaction (Aso *et al.*, 1974a,b). As a result of the incorporation of such hydrophobic amino acids, the product is expected to become rich in hydrophobicity and to be easily eliminated from the reaction system. This is considered to serve as a driving force for the reverse reaction to proceed efficiently.

In order to prepare a hydrophilic product, we then tried to incorporate glutamic acid by means of the plastein reaction. For the reason mentioned above, it may be necessary for glutamic acid to be in a hydrophobic form prior to starting the plastein reaction. In order to confirm this point we compared reactivities of three derivatives: Glu-α,γ-OEt_2, Glu-α-OEt, and Glu-γ-OEt. Free L-glutamic acid was also used for comparison. When a 2:1 (w/w) mixture of a peptic hydrolysate of soybean globulin and Glu-α,γ-OEt_2 was subjected to the plastein reaction, the resulting plastein yield was distinctly higher than any other cases, as expected; neither Glu-α-OEt, Glu-γ-OEt, nor free glutamic acid seemed to be effective in enhancing the plastein yield (Table I). As seen from the glutamic acid contents in the plasteins, Glu-γ-OEt was quite unreactive as was free glutamic acid. Glu-α-OEt was somewhat reactive, giving a better result than the above two cases. The best result was obtained with Glu-α,γ-OEt_2; the glutamic acid content approximately doubled (Table I). The amino acid compositions of several plasteins are shown compared with their materials (peptic hydrolysate of soybean globulin) (Table II).

The glutamic acid enriched plastein (Glu-plastein) was evaluated for water solubility. The evaluation was made in comparison with the control plastein (Table I) and with a soybean protein prepared according to Fukushima (1969). Table III shows results with water solubilities as a function of pH. The pH-solubility relation observed for the soybean protein is quite similar to that reported by Smith and Circle (1938). The control plastein showed an isoelectric precipitation as well, but the precipitation did not completely disappear in a higher or a lower pH region.

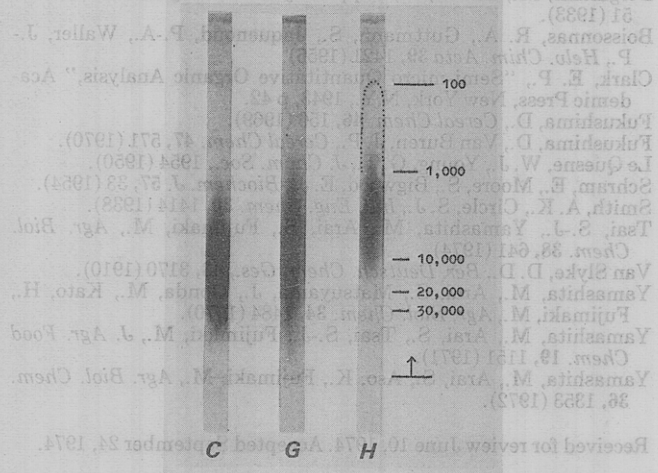

Figure 1. Polyacrylamide gel electrophoresis of plasteins and a peptic hydrolysate of soybean globulin: H, hydrolysate (av mol wt, 1280); G, glutamic acid enriched plastein (av mol wt, 6240); and C, control plastein (av mol wt, 12,050). The position of a low-molecular fraction unstainable with Amido Black 10B was found out by transcription with a filter paper and by treatment with ninhydrin. This Amido Black negative, ninhydrin positive position is indicated with dots in part H. On the right is shown a molecular weight scale estimated from the migration distances of several makers (Tsai *et al.*, 1974).

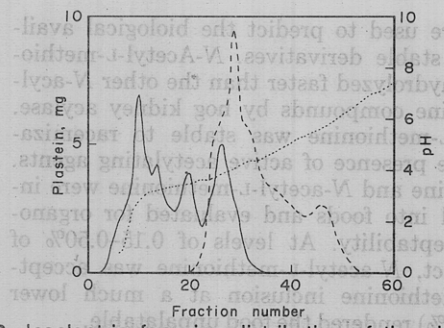

Figure 2. Isoelectric focusing distribution of the glutamic acid enriched plastein (solid curve) and the control plastein (broken curve). The dotted curve shows a pH gradient.

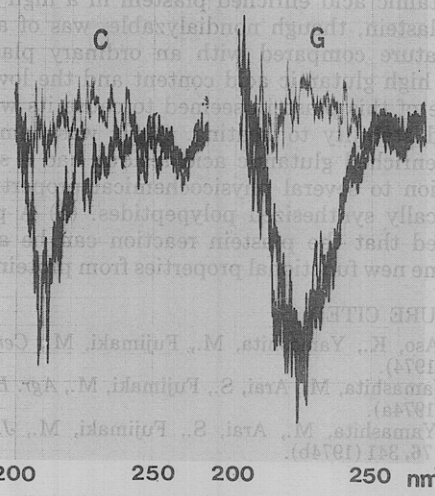

Figure 3. Circular dichroism spectra of the glutamic acid enriched plastein (G) and the control plastein (C).

Table V. *In Vitro* **Digestibilities of Plasteins and Tastes of the Digests**

Enzyme	pH[a]	Plastein[b]	Digest., %	Taste score[c] Bitter	Brothy
Pepsin	1.5	G	66.7	1.00 ± 0.00	1.33 ± 1.33
		C	73.4	4.50 ± 3.50	1.00 ± 0.00
α-Chymo-trypsin	8.0	G	48.2	1.00 ± 0.00	1.00 ± 0.00
		C	71.6	4.50 ± 1.50	1.00 ± 0.00
Molsin	3.0	G	65.8	1.00 ± 0.00	5.00 ± 0.00
		C	74.4	1.33 ± 1.33	1.33 ± 1.33
Pronase	8.0	G	66.3	1.00 ± 0.00	4.33 ± 1.33
		C	82.0	1.33 ± 1.33	1.17 ± 0.83

[a] For other conditions see the description in the text. [b] G, glutamic acid enriched plastein (Glu-plastein); C, control plastein. See also Table I. [c] Mean ± standard deviation; 5, very strong; 4, strong; 3, middle; 2, weak; and 1, no taste.

Glu-plastein was solubilized at any pH, with only slight turbidity remaining between pH 3 and 5 (Table III).

The soybean protein in solution was unstable against heat treatment at 100° and insolubilized in part in such a time course as shown in Table IV. This pattern of insolubilization is almost similar to that reported by Fukushima and Van Buren (1970). The control plastein was not susceptible to heat, although this sample *per se* was partly insoluble in water. On the other hand, Glu-plastein was stable and its solution remained clear during the heat treatment (Table IV).

In order to explain the high water solubility of Glu-plastein, it was compared with the control plastein with respect to the following three properties: molecular weight, isoelectric point, and circular dichroism.

A polyacrylamide electrophoretic study demonstrated that the migration of the control was slower than that of Glu-plastein (Figure 1), indicating that the latter consisted of lower molecular weight polypeptides than the former. Average molecular weights obtained by the amino-terminal determination were approximately 6240 for Glu-plastein and 12,050 for the control (Figure 1).

An isoelectric focusing experiment showed that the isoelectric point of the control plastein lay in the range of pH 3.7–5.8, whereas Glu-plastein had several isoelectric zones in a more acidic range such as pH 1.5–4.0 (Figure 2). It is apparent that the increment in the glutamic acid content (Tables I and II) is responsible for the isoelectric point shift toward the acidic side.

A circular dichroism study with Glu-plastein in solution gave a spectrum having a clear trough at 222 nm, suggesting the partial occurrence of an oligomeric (probably hexameric) glutamic acid sequence which was capable of forming an α-helix structure (Figure 3).

Putting together the above results, we can ascribe the high water solubility of Glu-plastein first to its rather low molecular nature and, second, to the extremely high content of glutamic acid. Also, such a large amount of glutamic residues is considered to be effective in protecting the plastein chains against their hydrophobic self-assembly by heating (Aso *et al.*, 1974b).

An additional study was made concerning *in vitro* digestibilities of Glu-plastein compared with the control. The digestibilities by four proteases were somewhat lower in Glu-plastein than in the control (Table V). An interesting result to note is that the Glu-plastein digests in all cases have no strong bitterness as do the control plastein digests. It is also noted that Glu-plastein treated with a microbial protease (Molsin or Pronase) has a strong brothy taste (Table V). In this connection a study is under way to assess a useful value of such a Glu-plastein digest as a potent flavor enhancer.

CONCLUSION

The following conclusions can be made. (1) The use of L-glutamic acid α,γ-diethyl ester was effective in synthesiz-

ing a glutamic acid enriched plastein in a high yield. (2) Such a plastein, though nondialyzable, was of a low molecular nature compared with an ordinary plastein. (3) Both the high glutamic acid content and the low molecular nature of this plastein seemed to cause its water solubility and stability to heating. (4) It was demonstrated that the enriched glutamic acid residue had a significant contribution to several physicochemical properties of the enzymatically synthesized polypeptides. (5) A possibility is expected that the plastein reaction can be applied to create some new functional properties from proteins.

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An Investigation of N-Substituted Methionine Derivatives for Food Supplementation

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N-Substituted methionine derivatives were prepared and evaluated as possible nutritional supplements for vegetable protein based foods. Ten of the C_1–C_{18} *N*-acyl derivatives of methionine, *N*-carbamoylmethionine (methionine urea), methionine hydantoin (cyclic urea), and *N*-carbethoxymethionine were prepared. All of the derivatives, except the carbethoxy- and methionine hydantoin, were stable to hydrolysis at pH 7, 100°. The remaining compounds were tested for reaction with reducing sugar models (Strecker degradation) and found to be stable relative to methionine at pH 5.5, 100°, for 1 hr. Isolated enzymes were used to predict the biological availability of stable derivatives. *N*-Acetyl-L-methionine was hydrolyzed faster than the other *N*-acyl-L-methionine compounds by hog kidney acylase. *N*-Acetyl-L-methionine was stable to racemization in the presence of active acetylating agents. L-Methionine and *N*-acetyl-L-methionine were incorporated into foods and evaluated for organoleptic acceptability. At levels of 0.15–0.50% of the product, *N*-acetyl-L-methionine was acceptable. L-Methionine inclusion at a much lower level (0.05%) rendered the food unpalatable.

The potential use of vegetable proteins to replace animal protein in food products has been well documented (*Chem. Eng. News*, 1971; Meyer, 1971; Hammonds and Call, 1970). It has been suggested that formulated foods, when replacing foods that make significant nutrient contribution, should at least equal the nutrient value of the food replaced (Council on Foods and Nutrition, 1968; Johnson, 1972). Vegetable proteins are deficient in sulfur amino acids, methionine and cystine. Addition of methionine to relieve this deficiency frequently makes the foods unpalatable for human consumption (Kies and Fox, 1971; FAO, WHO, UNICEF, Protein Advisory Group, 1970; Beigler, 1969). These flavor effects are caused by Maillard and Strecker degradation reactions, which yield volatile sulfides (Hodge, 1967). Methional has been identified as a major product from the Strecker degradation of methionine (Ballance, 1961). This reaction involves the amino group of methionine and proceeds *via* formation of an addition compound (hydroxyamine), followed by loss of water to yield an imine (Hodge, 1967). We have synthesized a series of methionine derivatives in which the amino group has been substituted with electrophilic groups so that the compound cannot undergo Strecker degradation. The chemical stabilities and enzyme stabilities of these compounds have been studied to evaluate them as replacements for methionine in food systems. The most promising derivative, *N*-acetyl-L-methionine, was tested for organoleptic acceptability.

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EXPERIMENTAL SECTION

Synthesis. All compounds prepared gave correct C, H, N elemental analyses and infrared and nmr spectra. Chemical purity was further established by a single spot for each compound on silica gel thin-layer chromatography plates. Solvent A (BuOH–H_2O–HOAc, 80:20:20) and solvent B (benzene–THF–HOAc, 70:30:1) were used for elution and iodine was used for development of the plates.

Acylmethionine compounds were prepared by three modifications of acylation reactions.

Method A: Reaction of Methionine with an Acid Anhydride. *Example: N-Acetyl-L-methionine.* L-Methionine (300 g, 2.0 mol) in 2 l. of water was adjusted to pH 10 with sodium hydroxide and cooled to 5°. Acetic anhydride (212 g, 2.1 mol) was added dropwise while pH 10 was maintained by addition of sodium hydroxide. After addition (~2 hr), the solution was stirred for 2 hr at 5–10° and allowed to reach room temperature. The solution was concentrated to 1 l. by vacuum evaporation, acidified to pH 1.0 with 6 *N* hydrochloric acid, and extracted with ethyl acetate. The organic layer was dried with sodium sulfate and evaporated under vacuum to yield a white solid. The crude product was recrystallized from 2 l. of acetone and 4