Plastein Reaction as a Method for Enhancing the Sulfur-Containing Amino Acid Level of Soybean Protein

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The plastein reaction, *i.e.*, a reversal of enzymatic proteolysis, was applied to prepare a higher-molecular, protein-like substance (plastein) in which the sulfur-containing amino acids supplemented were held in a state of peptide bonding. Soybean protein was hydrolyzed with pepsin and the hydrolysate (A) used as a material to be supplemented with methionine or half-cystine. A 50:50 mixture of ovalbumin hydrolysate (methionine source) and A was submitted to the plastein reaction with Nagarse (an alkaline proteinase produced by *Bacillus subtilis*). The resulting plastein contained methionine three times as much as A. Similarly, a plastein synthesized from a 50:50 mixture of wool keratin hydrolysate (half-cystine source) and A contained half-cystine seven times as much as A. L-Methionine derivatives also served as methionine sources. A plastein synthesized by use of papain from a 10:1 mixture of A and L-methionine ethyl ester showed a methionine content of 7.22%, this level being nearly six times that of A.

The plastein reaction is known to be an enzymatic process concerning the growth of peptide chains, *i.e.*, a reversal of the usual protein degradation by proteinases. The mechanism of such peptide-chain growth seems to involve the condensation and/or transpeptidation (Determann *et al.*, 1963; Haurowitz and Horowitz, 1955). A mixture of higher-molecular, protein-like substances formed from lower-molecular peptides (*e.g.*, protein hydrolysates) during the plastein reaction is called plastein. During the decades prior to 1960, many studies were carried out aimed at elucidating the relation between the plastein reaction and protein biosynthesis (Tauber, 1951; Wasteneys and Borsook, 1930). However, topical findings in 1957 of the amino acid-activating enzymes and aminoacyl-RNA's (Hoagland *et al.*, 1957; Holley, 1957; Ogata and Nohara, 1957) gave a clue to the modern aspect of the peptide-chain elongation *in vivo*. Thereafter, the plastein studies showed a rapid rate of decrease in quantity and, concurrently, were directed rather toward subjects of enzymological interest (Determann and Koehler, 1966; Wieland *et al.*, 1960).

We reinvestigated the plastein reaction, especially from the viewpoint of food science, and have provided much information basic to applying this reaction to protein food processing (Fujimaki *et al.*, 1970; Yamashita *et al.*, 1970a,b,c, 1971).

In the present paper we propose two methods for enhancing the sulfur-containing amino acid level of soybean protein, suggesting that the plastein reaction is applicable also to changing the nutritive values of proteins.

The formation of a peptide bond by condensation between free amino acids is a strongly endergonic process (ΔF = 4 kcal/mol). However, the free energy required to form a peptide bond from two peptides by condensation is far smaller (ΔF = 0.4 kcal/mol) (Fruton and Simmonds, 1958). Hence, the peptide chain growth during the plastein reaction is mostly attributable to the reaction among peptides; the contribution of dipolar amino acid ions is considered to be negligibly small, if any. On the other hand, the transpeptidation is essentially related to a reaction between peptides. Accordingly, as the sulfur-containing amino acid sources, for supplementation purposes, we used peptide mixtures such as an ovalbumin hydrolysate (rich in methionine) and a wool keratin hydrolysate (rich in half-cystine). This is the method described in this paper.

For the reason mentioned above, it may be impossible to alter clearly the amino acid composition, when free amino acids are used. However, as well as peptides, amino acid derivatives such as α-*N*-acyl amino acids, amino acid esters, and amides are useful since the negative free energy change in their hydrolysis is of sufficient magnitude to warrant the condensation as activated derivatives in the thermodynamic, if not in the kinetic, sense. The method described concerns methionine supplementation employing L-methionine derivatives.

MATERIALS AND METHODS

Protein Hydrolysates. The denatured soybean protein (alkali-extracted, acid-precipitated fraction) was incubated with pepsin (Sigma Chemical Co.) under the following conditions: substrate concentration, 1%; enzyme-substrate ratio (E/S), 1/100; pH, 1.6; temperature, 37° C; and incubation time, 24 hr. The resulting product was freeze-dried to obtain a powdered soybean protein hydrolysate (A). A commercial ovalbumin preparation (Difco) was treated in the similar manner to obtain a powdered ovalbumin hydrolysate (B). On the other hand, commercially available wool was previously treated with sufficient 3 *N* HCl at 100° C for 1 hr, and the product washed with 50% aqueous ethanol, 99% ethanol, and ether to obtain a wool keratin. The peptic hydrolysis of this keratin under the above-mentioned conditions and the subsequent freeze-drying yielded a powdered wool keratin hydrolysate (C).

Plastein Synthesis 1. A 50:50 mixture of A and B and a 50:50 mixture of A and C were used as the substrates of the plastein reaction. Nagarse (Nagase Sangyo Co.), a commercial preparation of an alkaline proteinase produced by *Bacillus subtilis*, was used as the plastein-synthetic enzyme. The optimum pH of this preparation for plastein synthesis

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Table I. Amino Acid Composition of Protein Hydrolysates and Plasteins

Amino acid	Soybean protein hydrolysate (A) %	Ovalbumin hydrolysate (B) %	Wool keratin hydrolysate (C) %	Plastein AB (from a 50:50 mixture of A and B) %	Plastein AC (from a 50:50 mixture of A and C) %
Arginine	7.45	4.68	5.64	6.14	6.46
Histidine	2.66	2.09	1.43	2.44	1.39
Isoleucine	5.20	7.25	4.61	6.36	5.00
Leucine	6.73	11.90	9.76	9.33	8.15
Lysine	5.81	5.23	2.18	5.45	3.32
Phenylalanine	4.29	7.01	2.58	6.33	3.83
Threonine	3.58	3.75	3.96	3.63	4.02
Tryptophan	1.34	2.00	2.51	1.77	2.61
Valine	4.97	8.42	6.90	7.91	6.66
S-Containing Amino acid[a]	**3.03**	**6.35**	**12.90**	**4.33**	**8.53**
Methionine	**1.25**	**3.92**	**0.70**	**2.33**	**1.10**
Half cystine	**1.78**	**2.43**	**12.20**	**2.00**	**7.43**
Alanine	4.08	5.15	7.15	4.59	6.22
Aspartic acid	11.51	8.41	4.54	8.94	6.37
Glutamic acid	16.94	9.18	10.13	12.21	12.20
Glycine	4.88	3.63	1.95	3.67	2.77
Proline	6.27	1.72	2.95	2.27	2.86
Serine	5.45	5.61	5.19	5.39	5.13
Tyrosine	3.34	4.88	2.70	4.45	3.19

[a] Methionine plus half cystine.

has been reported to be 6.0 (Yamashita *et al.*, 1971). Each substrate described above was dissolved in water and the solution incubated with Nagarse under the following conditions: concentration of substrate in aqueous solution, 30% (w/v); E/S, 2/100; pH, 6.0; temperature, 37° C; and incubation time, 48 hr.

Methionine Derivatives. *N*-Acetyl-L-methionine (Ac–Met), L-methioninamide (Met–NH_2), and L-methionine ethyl ester (Met–OEt) were prepared by the methods of Späth and Lederer (1930), Koenigs and Mylo (1908), and Boissonas *et al.* (1956), respectively. L-Methionyl-L-methionine (Met–Met), which was regarded as an activated derivative of methionine, was synthesized by the *N*-carboxyanhydride method (Bailey, 1967).

Plastein Reaction 2. A 10:1 mixture of A and one of the methionine derivatives was incubated under the above conditions (*cf.* plastein reaction 1) using one of the following enzymes: ficin (Tokyo Kasei Co.), Molsin (Seishin Seiyaku Co.), Nagarse (Nagase Sangyo Co.), papain (Sigma Chemical Co.), and Pronase (Kaken Kagaku Co.). In the case of the ficin- and papain-catalyzed reactions, 0.01 *M* L-cystein was used as the activator.

Determination of Constituent Amino Acid Contents. Sample (hydrolysate or plastein, 1 mg) was treated with 1 ml of 6 *N* HCl at 110° C for 20 hr, and the released amino acids quantified using an Hitachi amino acid analyzer KLA-3A. On the other hand, methionine and half-cystine were determined by the method of performic acid oxidation followed by 6 *N* HCl hydrolysis (Schram *et al.*, 1954), and tryptophan determined by the method employing dimethylaminobenzaldehyde (Spies and Chambers, 1948).

Feeding Test. Male rats (five rats per test) of the Wistar strain, initially weighing, on the average, about 50 g, were fed on the diet of the following composition: nitrogen source (N × 6.25), 10%; starch, 57.2–57.7%; sucrose, 15.0%; cellulose powder, 5.0%; oil, 5.0%; salts, 4.0%; vitamins, 0.85%; and choline hydrochloride, 0.15%. The ingredients of the salts and vitamins were similar to those proposed by Harper (1959). The protein efficiency ratio (PER) was obtained based on the average weight gain and nitrogen intake during 20 days feeding at 20° C.

RESULTS AND DISCUSSION

Supplementation Employing Peptide Mixtures. A whole incubation mixture after plastein reaction was dialyzed, using a cellophane membrane, against flowing water at 5° C for 4 days, to obtain a plastein (nondialyzable fraction) which was completely free from low-molecular substances such as amino acids, oligopeptides, and salts, and had neither taste nor odor. The ratio, on a dry basis, of the nondialyzable fraction *vs.* the whole incubation mixture, was represented in terms of plastein yield. Although the soybean protein hydrolysate (A), ovalbumin hydrolysate (B), and wool keratin hydrolysate (C) *per se* contained, respectively, 18.3, 26.9, and 39.3% nondialyzable fraction, the yields of plastein AB (plastein from a 50:50 mixture of A and B) and plastein AC (plastein from a 50:50 mixture of A and C) were 76.0 and 75.6%, respectively.

Table I shows the amino acid compositions of plasteins AB and AC together with those of A, B, and C. A characteristic point seen in this result is that, in amino acid pattern,

Table II. Protein Efficiency Ratios Evaluated in Rats

Dietary nitrogen source	PER (avg ± s.e.)
Casein[a]	2.40 ± 0.05
Soybean protein[b]	1.20 ± 0.20
Soybean protein hydrolysate[c]	1.28 ± 0.18
1:2 Mixture of plastein AC[c] and soybean protein[b]	2.86 ± 0.10
1:3 Mixture of the alkali-saponified plastein[d] and soybean protein[b]	3.38 ± 0.08

[a] Kanto Kagaku Co. (Japan). [b] The alkali-extracted, acid-precipitated fraction described in text. [c] *cf.* Table I. [d] The plastein (nondialyzable fraction) synthesized, using papain, from the soybean protein hydrolysate mixed with Met-OEt, and saponified under the alkaline conditions described in text.

Table III. Methionine Contents in Plasteins

Plastein-synthetic enzyme	Plastein yield[a] (%) (avg ± s.e.)	Methionine content[b] (%)				
		Methionine source[c]				
		Methionine	Ac–Met	Met–NH_2	Met–OEt	Met–Met
Ficin	71.8 ± 5.2	1.15	1.44	1.57	2.10	1.50
Molsin	68.3 ± 4.9	1.05	1.47	1.32		
Nagarse	75.0 ± 5.1	1.18	1.53	2.09	3.87	2.70
Papain	78.2 ± 3.6	1.20	1.22	3.12	7.22	5.10
Pronase	70.1 ± 4.0	1.15	1.32	1.43		
None		1.25	1.22	1.25	1.23	1.27

[a] The average plastein yield (percent of the nondialyzable fraction *vs.* the whole incubation mixture) was, in each case, not significantly influenced by the difference in the methionine source. [b] As an average value from duplicated experiments in each case. [c] The abbreviations are Ac–Met = *N*-acetyl-L-methionine, Met–NH_2 = L-methioninamide, Met–OEt = L-methionine ethyl ester, and Met–Met = L-methionyl-L-methionine.

both plastein AB and plastein AC are in proximity to an average of A and B, and to an average of A and C. A similar relation was observed also for the sulfur-containing amino acid contents as shown in Gothic style in Table I.

A PER evaluation was conducted with plastein AC. In order to adjust the sulfur-containing amino acid level to approximately 5%, this plastein was mixed with a two-fold-amount of the soybean protein (alkali-extracted, acid-precipitated fraction described already). Since the amino acid composition of this soybean protein was similar to that of A (Table I), the 1:2 mixture of plastein AC and soybean protein contained 1.20% methionine and 3.67% half-cystine. Table II shows the PER of this mixture in comparison with PER's of the controls (A, soybean protein, and commercially available casein of reagent grade). This result indicates that the wool keratin hydrolysate (C) used as a half-cystine source adequately contributes to decreasing the methionine insufficiency of the soybean protein (or hydrolysate) and to approximating its nutritive value to that of casein with the aid of the plastein reaction. It has been known that the half-cystine present in a diet at a high level acts as a sparer of dietary methionine because cystine has a methionine-sparing effect due to its feedback inhibition against the cystathionine synthetase activity (Rama Rao *et al.*, 1961).

Supplementation Employing Methionine Derivatives. Table III shows the methionine content in the plasteins (nondialyzable fractions) from the varying cases of combination. Free L-methionine added to the plastein-reaction system was quite unreactive as expected already; the values in the first column of this table were not larger than those in the lowest row (enzyme-free). However, Ac–Met and Met–NH_2 were, in most cases, effective in enhancing the methionine levels of plasteins. Met–OEt and Met–Met were found to be more effective derivatives. Especially, by use of papain as the enzyme, Met–OEt added to the soybean protein hydrolysate resulted in yielding a plastein containing 7.22% methionine in average. The data in Table III mean exclusively the amounts of methionine constituting the plasteins. Any trace amounts of free methionine and its derivatives employed were undetectable when each plastein was directly submitted to the amino acid analyzer.

In order to confirm that the methionine molecules were held in the plastein as its peptide-bond constituents, a degradation probe was carried out using a peptidase. The plastein (200 mg), synthesized with the aid of papain, from the 10:1 mixture of soybean protein hydrolysate and Met–OEt was previously treated with a mixture of ethanol (40.5 ml) and 2 *N* NaOH (4.5 ml) at 25° C for 3 hr to obtain an alkali-saponified plastein. This plastein was incubated with carboxypeptidase A (Sigma Chemical Co.) under the conditions described by Fraenkel-Conrat *et al.* (1955). In Figure 1 the time course is shown of the amino acid liberation during the incubation. The pattern of the methionine liberation curve indicates that the methionine held in the plastein is, as well as other constituent amino acid residues, in such state as is liberated only by treatment with the peptidase. The fact that the supplemented methionine in the plastein is not in free state is of special importance in view of the food processing and cooking, as mentioned already in this paper.

A PER evaluation in rats was conducted also with the above alkali-saponified plastein. However, this plastein was, because of its high methionine content (7.22%), diluted with a three-fold amount of the soybean protein (alkali-extracted, acid-precipitated fraction), to prepare a sample containing 2.74% of methionine. Table II (the last line) shows the PER evaluated with this sample, the value being nearly three times as much as that of the soybean protein or its hydrolysate.

By use of an appropriately controlled amount of Met–OEt it may be possible to prepare a plastein *per se* containing methionine at the above or more desirable level. Probably other methionine derivatives also can be effective sources of methionine, if their amounts are well controlled on use.

Unlike those, free methionine is, as expected, ineffective

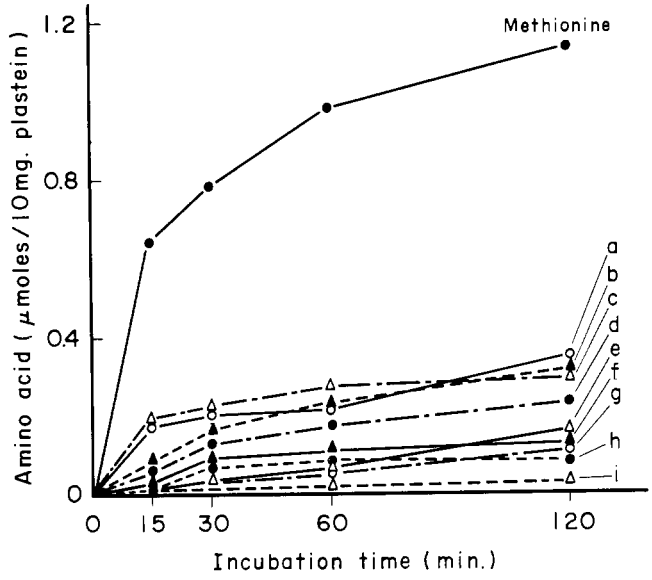

Figure 1. Time-course of amino acid liberation from the alkali-saponified plastein by treatment with carboxypeptidase A. a, phenylalanine; b, valine; c, leucine; d, alanine; e, a mixture of asparagine, glutamine, serine, and threonine; f, tyrosine; g, glutamic acid; h, isoleucine; and i, aspartic acid

in altering the amino acid patterns of plasteins. In general, even if a free amino acid is added to the system in large excess, its contribution to the plastein synthesis in view of kinetics is considered to be insignificant for the following reason. When a free amino acid (a) is condensed to a certain peptide (P) by mass action, with formation of higher-molecular peptide species ($P - a_1$, $P - a_2$, ..., $P - a_n$), their total concentration at equilibrium is given by

$$\Sigma[P - a_n] = [P]\Sigma[a]^n K^n$$

where K is the equilibrium constant for condensation. The free energy change accompanied with this type of condensation, *e.g.*, with the synthesis of an exterior peptide linkage, should lie in the midst of the free energy change for synthesizing an interior peptide linkage (ΔF: *ca.* 0.4 kcal/link) and that for synthesizing a dipeptide (ΔF: *ca.* 4 kcal/link). Taking this as 2 kcal for simplicity of calculation, K is roughly estimated as 0.03 (at 27° C). Assuming that [P] and [a] are kept at 0.1 *M* and 1 *M*, respectively, we can obtain the following results concerning the concentrations of the yielded peptides: $P - a_1$, 3×10^{-3} *M*; $P - a_2$, 9×10^{-5} *M*; $P - a_3$, 27×10^{-7} *M*; and so on. If we apply 1500 to P as its molecular weight, by analogy of the average molecular weight observed for a peptic hydrolysate of soybean protein (Yamashita *et al.*, 1970c), the result is that 0.1 mol of P and 1 mol of methionine (m), for example, react with each other to produce a peptidylmethionine or methionylpeptide ($P - m_1$) in the yield of about 5 g. The share of the methionine residue in $P - m_1$ is approximately 9% on a weight basis. Accordingly, it is found that the methionine in a bound state results in at most 0.3% of a total weight of the peptides in the system. This appears to rule out the possibility of the effective alteration of amino acid composition through incorporating free methionine by mass action, even in the presence of an enzyme to bring the system into equilibrium. Thus the plastein reaction is a process relating, in practice, to activated amino acid derivatives.

In order to obtain further evidence for securing the superior reactivity of the methionine derivatives to free methionine, the pK' values were measured employing an automatic titrator, according to the method of Tanford and Hauenstein (1956). The results were as follows: free methionine, $pK_1' = 2.3$ and $pK_2' = 9.2$; Ac-Met, $pK_1' = 3.7$; Met-NH_2, $pK_2' = 7.8$; Met-OEt, $pK_2' = 7.5$; and Met-Met, $pK_1' = 3.2$ and $pK_2' = 7.5$. Between free methionine and any of these derivatives it was found that there were differences of about 1 in pK_1' and of about 1.5 in pK_2'. Such differences may warrant the more condensability of the amino and/or carboxyl groups of the methionine derivatives.

CONCLUSION

Using appropriate derivatives, including peptide mixtures, as sulfur-containing amino acid sources, it is possible to revise the nutritive value of soybean protein through its enzymatic modifications (peptic hydrolysis and subsequent plastein synthesis); the resulting product, *i.e.*, plastein, is not only nutritious but also, probably, acceptable as a protein-like food.

It is expected that this enzymatic method may be applicable even to altering the general amino acid compositions of proteins. Of importance for this purpose is the matter of selecting and developing the enzymes and amino acid derivatives (including peptide mixtures) specific to synthesizing the plasteins of desirable amino acid patterns.

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