Location and State of Methionine Residues in a Papain-synthesized Plastein from a Mixture of Soybean Protein Hydrolysate and L-Methionine Ethyl Ester†

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With the aid of papain, a plastein was synthesized from a 1:10 mixture of L-methionine ethyl ester and a peptic hydrolysate of soybean protein. Dialysis of the whole reaction-product yielded a methionine-incorporated plastein (Met-plastein) as the nondialyzable fraction, its yield being 78.2% on a dry-matter basis, of the whole reaction-product. Methionine content in this Met-plastein was 7.22% on a weight basis, while its content in the material hydrolysate was only 1.25%. Carboxypeptidase A treatment of Met-plastein liberated methionine at an outstandingly rapid rate. A similar, but not so outstanding, rate was observed for the methionine liberation from Met-plastein by treatment with leucine aminopeptidase. Methyl isothiocyanate treatment and subsequent cyclization yielded a mixture of methylthiohydantoin from Met-plastein. Gas chromatographic analysis of this mixture after trimethylsilylation showed a result that methionine occupied 33.2%, on a molar basis, of the total N-terminal amino acids. Lithium borohydride reduction and 6N hydrochloric acid hydrolysis of Met-plastein produced monomeric aminols, and their 2,4-dinitrophenylation followed by thin-layer chromatography gave a result that methionine occupied 84.9%, on a molar basis, of the total C-terminal amino acids; the residues amounting to 14.4% of the C-terminal methionine residues remained as an ethyl ester form. The selective degradation probe employing cyanogen bromide to generate free homoserine disclosed that the occurrence of the polymeric methionine-methionine sequence was little if any in Met-plastein. Based on the above experiments as well as an evaluation of the esterase activity against L-methionine ethyl ester, a possible mechanism was discussed of the papain-catalyzed synthesis of plastein in a system containing such an ester.

Whilst a series of our previous works were relevant to producing a bland, protein-like foodstuff in terms of plastein,^{1~5)} the preceding short communication dealt with a method of enhancing the sulfur-containing amino acid level of soybean protein by use of the plastein reaction.⁶⁾ Its detailed article has been published elsewhere.⁷⁾ Especially in the methionine enhancement by such an approach, the most satisfactory result has been obtained

in the case where the reaction is made with L-methionine ethyl ester (Met-OEt) as a methionine source to be added to the soybean protein hydrolysate (substrate) and with papain as a plastein reaction catalyzer. The above papers^{6,7)} have reported that the methionine incorporated through the plastein reaction is of a peptide-bonding state and its content in the resulting plastein is 5.25~9.19% on a weight basis.

In the present paper we elucidate the positions and states of the methionine residues in the plastein synthesized from a mixture

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of a soybean protein hydrolysate and Met-OEt with the aid of papain, and discuss a possible mechanism of the papain-catalyzed plastein reaction in the system containing Met-OEt.

MATERIALS AND METHODS

Enzymes. The following proteinases were used: a recrystallized preparation of pepsin (Sigma Chemical Co.) for the hydrolysis of the soybean protein (described below) and a purified preparation of papain (the same source) for the subsequent synthesis of plastein.

Substrates. An alkali-extracted, acid-precipitated fraction from soybean protein was used as a substrate and its hydrolysis carried out with pepsin under the conditions described in the preceding papers.^{6,7} The resulting degree of hydrolysis probed by the conventional trichloroacetic acid treatment was approximately 80% and the average molecular weight determined based on the Van Slyke's method (procedures appearing also in the present paper) was 1,550.^{6,7} A 10:1 mixture (on a weight basis) of the above hydrolysate and a crystalline preparation of L-methionine ethyl ester (Met-OEt) was used as a substrate of the plastein reaction.

Preparation of L-methionine-incorporated plastein (Met-plastein). The above substrate was incubated with papain, together with L-cysteine as a papain-activator, under the conditions mentioned in the preceding papers.^{6,7} The whole mixture after incubation was dialyzed through a cellophane membrane against running water at 5°C for 4 days, and the nondialyzable fraction in terms of Met-plastein was obtained (yield: 78.2 g from 100 g of the whole incubation mixture on a dry-matter basis). No significant amount of free methionine was detectable in Met-plastein by its direct analysis with an amino acid analyzer (Hitachi KLA-3B). It was apparent that any monomeric Met-OEt was also absent in Met-plastein, since DNP-methionol was undetected in the product derived from Met-plastein by reduction with LiBH₄ and by subsequent dinitrophenylation with 1-fluoro-2,4-dinitrobenzene (FDNB) (experimental conditions described below).

Estimation of average molecular weight. The usual Van Slyke's method was applied to determine the

amino nitrogen content in Met-plastein. The value obtained was corrected by the content of lysine- ϵ -amino nitrogen. Based on the corrected value, the average molecular weight was calculated on the assumption that the plastein molecule shaped as a polypeptide bearing one N-terminus.

Leucine aminopaptidase (LAP) treatment. A suspension of 20 mg Met-plastein in 10 ml of 0.005 M MgCl₂-0.005 M Tris buffer (pH 8.5) was incubated with 1 mg of LAP (Sigma Chemical Co.), according to the conditions proposed by Hill and Smith.⁸ Samplings were made in the course of incubation, and each sample was immediately mixed with an equal volume of 0.2 M citrate buffer (pH 2.2) to stop the enzymatic reaction. Each mixture was directly submitted to the amino acid analyzer to determine the liberated amino acids.

Carboxypeptidase A (CPase A) treatment. To hydrolyze the C-terminal methionine ethyl ester, Met-plastein was treated with a mixture of ethanol (40.5 ml) and 2 N NaOH (4.5 ml) at 25°C for 3 hr. A suspension of 40 mg of the alkali-hydrolyzed Met-plastein in 10 ml of 0.1 M carbonate (pH 8.5) was incubated with 0.4 mg of CPase A (Sigma Chemical Co.), according to the conditions proposed by Fraenkel-Conrat *et al.*⁹ The subsequent procedures were similar to those mentioned above.

Determination of N-terminal methionine. The method of Vance and Feingold¹⁰ was used to split the N-terminal amino acids with formation of methylthiohydantoin (MTH) derivatives. From the reaction mixture these derivatives were extracted with sufficient ethyl acetate and the extract was treated with *N,O*-bis (trimethylsilyl)acetamide-acetonitrile (1:3, v/v) to obtain a mixture of trimethylsilyl (TMSi) derivatives of MTHs. The content of TMSi MTH-methionine in this mixture was determined by gas chromatography. A Simadzu gas chromatograph GC-4A, equipped with a 3 m column of 1% OV-17 and with a flame ionization detector, was used, and its operation carried out during 160°C~260°C at the programming rate of 4°C per min (retention time: 8.5 min for TMSi MTH-methionine). The authentic sample prepared similarly from crystalline L-methionine was also gas-chromatographed to obtain a dose-response relation.

Determination of C-terminal methionine. A given amount of Met-plastein was methylated by the method

of Chibnall *et al.*¹¹⁾ and subsequently reduced with LiBH_4 under the conditions proposed by Chibnall and Rees,¹²⁾ to convert the C-terminal amino acids to corresponding aminols. The reaction product was dialyzed through a cellophane membrane against running water to remove the reagents used and their reactants, and the nondialyzable fraction freeze-dried to obtain a dehydrated powder. A mixture of this powder (15 mg) and 6 N HCl (10 ml) was placed in a test tube, degassed below 10^{-4} mmHg by the freeze and thaw technique employing liquid nitrogen, and treated at 110°C for 24 hr to liberate monomeric aminols. Without separating aminols from amino acids liberated together, the whole reaction product was treated with FDNB by the Sanger's DNP-method.¹³⁾ An excessive amount of glycine was added to the reaction mixture after dinitrophenylation to eliminate unreacted FDNB. DNP-aminols were extracted with ether from the reaction mixture (slightly alkaline) and the extract was washed repeatedly with 1% NaHCO_3 to remove DNP-amino acids. An aliquot of the extract was spotted on a silica gel thin-layer and developed with chloroform-acetone (19:1). A zone ($R_f=0.75$), in agreement with that of authentic DNP-methionol (mp $140\sim 141^\circ\text{C}$) prepared likewise from crystalline L-methionine, was extracted with a given amount of redistilled chloroform. The optical density of the extract was measured at $340\text{ m}\mu$ by use of an Hitachi recording spectrophotometer ($\epsilon=1.06\times 10^4$ for authentic DNP-methionol).

Determination of C-terminal methionine ethyl ester. A mixture of Met-plastein (100 mg) and 0.1 N NaOH (2 ml) was placed in a sealed test-tube and allowed to stand overnight at room temperature with intermittent shaking. An aliquot of this mixture was then pipetted and diluted with an equal volume of water containing *n*-propanol ($5\text{ }\mu\text{l}/100\text{ ml}$) as an internal standard. The diluted suspension was centrifuged and the supernatant analyzed for ethanol by gas chromatography under the following conditions: apparatus, the same described already; solid phase, 10% carbowax 20 M; column size, $4\text{ mm}\times 3\text{ m}$; and column temperature, 80°C (retention times: 7.5 min for ethanol and 12.5 min for *n*-propanol). For calibration, an aqueous solution of known concentration of ethanol and *n*-propanol was analyzed similarly.

Selective degradation probe for peptide bond involving methionine residue. To 100 mg of sample in 2 ml of 70% formic acid was added 1.2 g of BrCN prepared

by the method of Shimizu.¹⁴⁾ This mixture was held for 48 hr at room temperature with mild stirring, according to Corradin and Harburg.¹⁵⁾ Subsequently, the reaction mixture was freeze-dried to obtain a powdered product. To an aliquot (50 mg) of this product was added 1 ml of 0.4 M pyridine-acetate buffer (pH 6.5), and the mixture was treated under the conditions (110°C , 1 hr) proposed by Ambler and Brown,¹⁶⁾ to hydrolyze the lactone ring. As described below, 1 mole of methionylmethionine residue can yield 1 mole of monomeric homoserine through the above reactions, when these proceed stoichiometrically. Thus, the hydrolysate was directly submitted to the amino acid analyzer to detect and determine homoserine. To determine a total amount of free and bound (*i.e.* C-terminal) homoserine, the mixture was hydrolyzed with 6 N HCl at 110°C for 20 hr and the resulting hydrolysate was submitted to the amino acid analyzer.

Esterase activity assay with Met-OEt as substrate.

The above mentioned preparation of papain (Sigma Chemical Co.), having an esterase activity of 3.75 $\mu\text{mole}/\text{min}/\text{mg}$ in BAEE unit, was used. This papain preparation (0.3 mg) was dissolved in 0.5 ml of 0.05 M acetate buffer (pH 5.0) containing 0.001 M NaCN (papain activator) and 0.015 M *n*-propanol (internal standard for gas chromatography), and the solution preincubated at 37°C for 1 hr. On the other hand, Met-OEt (30 mg) was dissolved in 0.5 ml of the same buffer containing NaCN and *n*-propanol likewise, and the solution held at 37°C . Both solutions were combined with each other in a test tube with a stopper, and the resulting mixture incubated at 37°C with mild stirring. In the course of the incubation, aliquots were sampled by use of a microsyringe and immediately analyzed for ethanol (also for *n*-propanol) by gas chromatography; its conditions of operation were similar to those described in the paraphrase of the determination of C-terminal methionine ethyl ester.

RESULTS AND DISCUSSION

The plastein reaction conducted in the present work yielded, on a dry-matter basis, 78.2 g Met-plastein (nondialyzable fraction) from 100 g of the 10:1 mixture of the soybean protein hydrolysate and Met-OEt, its methionine content being 7.22% on a weight basis. Since the content of methionine *per se* occur-

ring in the soybean protein hydrolysate is 1.25% on a weight basis, the amount of methionine consumed to form Met-plastein is calculated to be approximately 70% on a molar basis.

LAP and CPase A treatments of Met-plastein led to the methionine liberation as in Fig. 1. The patterns of its liberation (A and B) indicated that methionine occurred in Met-plastein in such a state as is liberated as a result of its hydrolysis with the peptidase. Apparently, this means that the state of methionine is peptide-bonding. On the other hand, the full possibility that the methionine residues in such a state in Met-plastein are hydrolyzable by intestine enzymes has been demonstrated based on the bio-utility evaluation in rats.⁷⁾

Figure 1 further suggests that the methionine residues localized probably at both termini of Met-plastein chains. The difference between the patterns A and B concerning the methionine liberation rate may predict that the C-termini bear more methionine residues than the N-termini.

Table I shows the shares of the methionyl and methionine residues in Met-plastein in comparison with those in the control (an ordinary plastein from a peptic hydrolysate, without Met-OEt, of soybean protein); the values listed therein are those obtained in the following manner. The average molecular

weight of Met-plastein was determined as 9,690. On the other hand, an average molecular weight of the amino acid residues in

FIG. 1. Liberation of Amino Acids from Methionine-incorporated Plastein by Treatment with Leucine Aminopeptidase (A) and with Carboxypeptidase A (B).

TABLE I. SHARES OF METHIONINE RESIDUES IN THE METHIONINE-INCORPORATED PLASTEIN

Sample	Location of residue	Weight percent	Moles of residue per 1 mole plastein	Molar probability of share (%) per amino acid residue	
Methionine-incorporated plastein	N-Terminal	0.45	0.332	33.2	{ 72.7 ... Carboxyl form 12.2 ... Ester form
	C-Terminal	1.15	0.849	84.9	
	Interior	5.62 ^{a)}	4.204 ^{a)}	4.8 ^{a)}	
	Total	7.22	5.385	—	
Control ^{b)}	Total	1.25	1.211	1.3	

^{a)} Calculated value.

^{b)} An ordinary plastein from a peptic hydrolysate, without Met-OEt, of soybean protein. The average molecular weight of this plastein was assumed to be equal to that of Met-plastein.

Met-plastein is calculable from its amino acid composition on a molar basis. Taking this as 108 for convenience, we can obtain 90 as a rough estimate of the degree of polymerization of Met-plastein. Supposing that each Met-plastein molecule shapes as a one-head, one-tail polypeptide of molecular weight 9,690 and, therefore, of 90 residues, a weight-mole interrelation as listed in Table I is obtainable of a methionine residue type in question. Thus, the values observed experimentally for the N- and C-terminal methionine residues (33.2 mole% and 84.9 mole%, respectively) are convertible to the amounts by weight percent (0.45% and 1.15%, respectively). Such difference in the methionine share between both termini may well explain the difference between the above-mentioned two patterns (A and B in Fig. 1) concerning the liberation rates of methionine relative to other amino acids. This result may suggest that Met-OEt is able to act both as an acylating reagent of papain and as a replacement amine of an acyl-papain, and also that the latter action predominates over the former one. On the other hand, the amount (on a weight basis) of the interior methionyl residues, *i.e.*, those situated between the second and eighty ninth positions from the head to the tail, is calculated by subtracting the sum of 0.45% and 1.15% from the total (7.22%). The resulting value, 5.62%, is equivalent to 4.204 moles methionyl residue per 1 mole Met-plastein, and also to molar probability 4.8% [= (4.204/88) × 100]. A significant amount in this case over the corresponding value (1.3%) in the control indicates that a number of Met-OEt molecules are participated not only in coupling with the termini of the initial or intermediate peptides but also in forming the interior peptide bonds of the intermediate or final products.

Most peptides and α -amino acid esters are considered to be similar each other in pK_2' -value.⁷⁾ It may be, therefore, possible that, as well as the peptides constituting the protein

hydrolysate, Met-OEt is capable of attacking the acyl-papain. The papain-catalyzed reaction is known to proceed with the aid of the cationic imidazole, *i.e.*, His-158, as a general acid catalyzer.¹⁷⁾ In aminolysis, the problem of the pK_2' -value of replacement amine is supposed to be somewhat more important to the papain-catalyzed reaction than to, for example, the α -chymotrypsin-catalyzed one where the replacement amine is activated through binding with His-57, a general base catalyzer.¹⁸⁾ Less methionine share at the N-termini than at the C-termini of Met-plastein (Table I) may suggest that the rate of formation of methionyl-papain is not so rapid as that of peptidyl-papain since the system contains almost equimolar concentrations of Met-OEt and peptides at the initial stage of reaction.

The selective degradation, employing BrCN, of a peptide bond involving the carboxyl side of an interior methionine residue produces a homoserine lactone terminus, and its lactone ring cleavage with the pyridine-acetate buffer gives rise to a peptide bearing homoserine at the C-terminus. Hydrolysis of this peptide with 6N HCl yields free homoserine which is determinable by use of the amino acid analyzer under the standard conditions; its position of elution is usually in the neighborhood of that of glutamic acid. However, a methionylpeptide, *i.e.*, a peptide bearing a methionine residue at the N-terminus, when treated with BrCN and subsequently with the pyridine-acetate buffer, produces free homoserine without being hydrolyzed with 6N HCl. On the other hand, this method is used to indicate the presence or absence of the polymeric methionine-methionine sequence.¹⁹⁾ A peptide bearing an interior methionylmethionyl residue, for instance, gives rise to a free homoserine, when treated by this method. Dissimilarly, 1 mole of an N-terminal methionylmethionyl peptide produces 2 moles of free homoserine by this treatment. Thus, a value obtained by sub-

tracting the amount of the N-terminal methionine from that of the free homoserine indicates the number of the methionine-methionine link. We found that this number was 0.083 on a molar basis per 1 mole of Met-plastein. This result suggests that the occurrence of the polymeric sequences of methionine is little if any.

In order to obtain another evidence that might partly warrant the difficulty of the occurrence of such polymeric sequences, we carried out an experiment of detecting L-methionyl-L-methionine in an incubation mixture of Met-OEt and papain. The conditions of the incubation was similar to those adopted in assaying the esterase activity of papain against Met-OEt, and an aliquot of the incubation mixture was submitted to a silica gel thin-layer to be developed with *n*-butanol-acetic acid-water (4:1:2) at room temperature. However, no appreciable spot showing the presence of this dipeptide was detected when the thin-layer was treated with the ninhydrin reagent (authentic L-methionyl-L-methionine: $R_f=0.65$). It may well be mentioned, therefore, that Met-OEt is hydrolyzed (as described later) without formation of methionine-methionine polymers.

Water-soluble peptides grow higher-molecular as the plastein reaction proceeds, and the products are finally eliminated from the reaction system as insoluble polymers. The molar fraction of Met-OEt may increase accordingly. Near the final stage of reaction, it may be possible that Met-OEt attacks peptidyl-papain with a higher frequency to yield a peptide bearing methionine, or its ethyl ester, at the C-terminus. A similar, but somewhat less frequent, attack by Met-OEt on papain may generate methionyl-papain on the other hand, the subsequent aminolysis leading to the formation of a peptide bearing methionine at the N-terminus.

Papain is known to be endowed with an esterase activity.^{20,21)} Although the standard method has been proposed to assay the esterase

activity by use of benzoyl-L-arginine ethyl ester (BAEE) at 25°C,²²⁾ we made an experiment with Met-OEt under the conditions relevant to those for the plastein reaction. Figure 2 shows a time course of the ethanol liberation as a result of the Met-OEt hydrolysis by papain at 37°C. With a lag phase of a few hours, the liberation developed rapidly and reached a plateau after 18 hr. At the stage of this rapid liberation, the esterase activity is calculated as 1.83 μ moles/min/mg papain. This result may be roughly comparable with 3.75 μ moles/min/mg in BAEE unit at 25°C which is a certified activity of the papain preparation used, although the experimental conditions including temperature differ from each other. At the final stage, it is observable that a molar fraction amounting to nearly 80% of the substrate (Met-OEt) has been hydrolyzed. This result indicates that methionyl-papain is easily formed immediately to leave the ethanol moiety. By analogy, it is considered that peptidylmethionine ethyl ester also suffers from hydrolysis by papain in a similar manner. This may be partly supported by the finding that most

FIG. 2. Hydrolysis of L-Methionine Ethyl Ester by Papain at 37°C.

of the C-terminal methionine residues of Met-plastein are not of an ester form (Table I). The acyl-papain can be attacked competitively either by water or by amines. It is likely that the hydrolysis predominates over the aminolysis at the final stage of the plastein reaction. On the other hand, there is a possibility that a part of higher-molecular peptidylmethionine ethyl ester is precipitated and eliminated from the system before formation of new peptidylmethionyl-papain. Such a possibility may explain the result that a small amount (14.4% on a weight basis) of peptides of the ester form remains even at the final stage of the plastein reaction.

In general, the alkaline hydrolysis of thiol esters shows notably slower rates than that of their oxygen counterparts. This may be reflected by the pH-rate profiles of "cystein" peptidolytic enzymes differing from those of "serine" peptidolytic enzymes. Moreover, thiol esters are more reactive than oxygen esters toward nitrogen nucleophiles and their aminolysis is accelerated catalytically by imidazole.¹⁷⁾ Blackburn²³⁾ analyzed the reaction mode of the imidazole-catalyzed aminolysis of methyl thioformate (a papain model) by glycine ethyl ester, and described that the reaction proceeded through general acid catalysis, rather than through nucleophilic one, below pH 6.5 where the rate of acylation of papain was maximal. Supposedly, it may be probable that the actual papain-catalyzed aminolysis shows some similar features. In this connection a pH-rate profile has been presented of the plastein synthesis with papain,⁵⁾ in which the maximum rate of synthesis is observed at pH 5. Such features of reaction are suggestive in considering the possible mechanism of the plastein synthesis by papain from the substrate constituted with peptides and an amino acid ester.

As for "serine" peptidolytic enzymes, papers will be presented in relevance to their acylation and aminolysis by lower-molecular peptides, to characterize the mechanism of the

plastein reaction.

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