Müller (1977) detected that in fresh carrot root phospholipid-bound choline amounts to 47–83 μg/g; this corresponds to 26–46 mg of PC/100 g, which is compatible with our results. Hölzl (1965) reported that of the PL of lyophilized carrot 20% was PE, 19% PC, 18% PI, and 9% cardiolipins, but at the same time as much as 27% PA and 7% polyphosphatidic acids. Evidently, different techniques in sample preparation and possibly in analysis explain part of the differences between this composition and our results.

Gregor (1977) obtained significantly lower lipid content (0.4–0.5 mg/g fresh weight) than that of other authors or our result, by repeated extraction of TCA homogenates from two carrot cultivars with chloroform–methyl alcohol mixture (2:1, v/v). In these extracts also the relative distribution of triglycerides and phospholipids (1:0.7) was different from that in our material.

Plasmalogens are common minor components of animal lipids but they have been detected also in certain plants as in pea seeds (Wagenknecht, 1957; Kaufman et al., 1970) and in different beans (Kuroda and Takahashi, 1975). In the present study the origin of aldehydes detected in the PL hydrolysate was not determined, but interpretation of plasmalogen nature of aldehydogenic PLs may be based on specificity and correct use of respective fractionation and analysis methods. The presence of aldehydogenic lipids in carrot suggests that small amounts of plasmalogens might occur also in other plants.

The total lipid content and the relative amounts of NL, GL, and PL, and also the composition of polar lipid fractions in carrot roots, appears in general very similar to that in many other nonphotosynthetic plant storage tissues containing little fat, such as sugar beet (Beiss, 1969), sweet potato (Walter et al., 1971), and potato tuber (Galliard, 1968). Although these equivalent tissues have very similar lipid composition, carrot root lipids differ significantly from carrot leaf lipids. Tevini (1976) reported that in green and yellow carrot leaves galactosyl diglycerides are predominant lipids, amounting to about 80% of polar lipids. Furthermore, the relative amount of MGDG and PG in leaf lipids is remarkably higher than that in root lipids. The essential differences between carrot leaf and root lipids are similar to general differences between the lipids of leaves and different nonphotosynthetic tissues in many plants (e.g., Hitchcock and Nichols, 1971).

The lipid pattern of carrot suspension culture cells grown at 25 °C in the dark (Kleinig and Kopp, 1978) differs from that in our root material most in the relatively low galactolipid content (6.4% of total lipids), evidently because of different plastid content. The composition of major PLs seems to be similar in root material and in cell cultures, but the latter contain less PG which is also typical to plastids (2% of total PL) and PS (trace amount).

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Papain-Catalyzed Synthesis of Methionine-Enriched Soy Plasteins. Average Chain Length of the Plastein Peptides

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A plastein was synthesized from a peptic soy protein hydrolyzate and ^{14}C-labeled methionine ethyl ester as substrates and with papain as a catalyst. The resulting water-insoluble plastein contained 14.3% of covalently linked methionine. By partial or complete oxidation of the plastein-linked methionine, it was converted into a water-soluble peptide mixture. This mixture was shown by gel chromatography on Sephadex G-25 to consist of peptide species of the same size as the substrate peptides used for plastein synthesis. Radioactive methionine had been incorporated into the entire range of substrate peptides. By chemical means the average chain length was established to be about six residues for both substrate and product peptides. No high-molecular-weight species were formed during plastein synthesis.

The plastein reaction is the protease-catalyzed conversion of soluble peptides into a water-insoluble product. The enzymatic reaction involves cleavage and resynthesis of peptide bonds. Synthesis of peptide bonds was claimed to occur by condensation of α-amino and α-carboxyl groups of peptides with elimination of water (Wieland et al., 1960; Yamashita et al., 1974), as well as by transpeptidation (Horowitz and Haurowitz, 1959).

For the food chemist it is of interest to know if plasteins are high-molecular-weight products or lower peptides. Is the enzymatic conversion from the soluble to the water-

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insoluble state related to an increase in molecular weight?

Yamashita et al. (1970) claimed that, during chymotrypsin-catalyzed plastein synthesis, the average chain length of the peptides increased by a factor of about 3. The same authors (Yamashita et al., 1974) stated in another publication that the molecular weight of peptides of a plastein produced with papain ranged between 2000 and 30 000 with an average of approximately 12 000. This seemed to be an indication that plastein was synthesized at least in part by condensation reactions giving rise to high-molecular-weight species from substrate peptides which had molecular weights below 1000.

In contrast to these findings, Hofsten and Lalasidis (1976) were unable to find a difference in the elution profile on Sephadex of a fish protein hydrolysate and the corresponding plastein.

We decided to reinvestigate the question of chain length in plasteins after we had discovered that plasteins enriched in methionine were completely soluble in dimethyl sulfoxide and that, by partial or complete oxidation of methionine to the sulfoxide or the sulfone, these plasteins became perfectly water-soluble peptide products.

EXPERIMENTAL SECTION

Enzymes. Twice crystallized pepsin (Sigma Chemical Co., No. P-7012) was used to prepare the substrate peptides for the plastein reaction. For plastein synthesis we used twice crystallized papain (Sigma Chemical Co., No. P-4762).

Substrate. Fifty grams of a commercial soy protein isolate with a total nitrogen content of 14.6% were digested at pH 1.6 and at a concentration of 5% total solids for 24 h at 37 °C with 1% by weight of porcine pepsin. The pH of the digest was then raised to 5.5 with 2 N NaOH prior to heating (15 min at 80 °C) in order to destroy the pepsin. The digest was allowed to stand at 4 °C overnight. A sediment was removed by centrifugation and the supernatant peptide solution was freeze-dried. We obtained 46 g of lyophilized product with a total nitrogen content of 12.8%. In order to remove excessive salt and the smallest of the peptides present in the digest, 10 g of the product was ultrafiltrated in an ultrafiltration cell equipped with an Amicon UM-05 membrane (nominal cut-off = 500 molecular weight). Subsequently, the concentrate was ultrafiltrated in another cell equipped with the Amicon UM-10 membrane (nominal cut-off = 10 000 molecular weight) in order to remove the largest of the peptide species present in the digest. The filtrate was collected and freeze-dried to give 7.8 g of a product with a total nitrogen content of 13.5%.

L-[^{14}C]Methionine Ethyl Ester Hydrochloride. L-[1-^{14}C]methionine (4.975 mg) with a specific activity of 402 μCi/mg was obtained from Amersham Radiochemicals, England. This product was mixed with 300 mg of cold L-methionine and esterified with the thionyl chloride procedure (Brenner and Huber, 1953). After recrystallization from ethanol–diethyl ether we obtained 240 mg of crystalline product. The radiochemical purity of our ethyl ester was estimated to be 93% by scanning of thin-layer plates. The remaining radioactivity was located within the methionine (free carboxyl) spot. For the purpose of plastein synthesis, the product was diluted with cold crystalline ethyl ester to a specific activity of ca. 300 μCi/mmol. A solution in ethanol containing 21.37 mg or 30 μCi/mL was prepared.

Plastein Synthesis. Substrate peptides (2.5 g) were dissolved together with 500 mg (2.34 mmol) of cold methionine ethyl ester hydrochloride in a few milliliters of an aqueous solution 10 mM in L-cysteine and 1 mM in EDTA. The pH was adjusted to 5.5 and the temperature of the reaction mixture equilibrated at 37 °C. One-hundred microliters (2.137 mg or 3.0 μCi) of L-[^{14}C]methionine ethyl ester in ethanol was injected. At this stage, the final concentration of solids was usually between 35 and 40%. Papain (25 mg), dissolved in a little aqueous L-cysteine (50 mM) and EDTA (5 mM), pH 7.0, was then injected. During the first hour of reaction, a steady and rapid drop in pH was compensated by injecting small volumes of 1 N NaOH. The reaction was then left to itself for another 23 h. The pH dropped to ca. 4.7 in this time.

Plastein Isolation. The plastein was suspended in 20 volumes of distilled water and briefly heated to 90 °C to destroy the papain. After cooling, the suspension was transferred to an ultrafiltration cell equipped with an Amicon UM-05 membrane. The suspension was concentrated under nitrogen pressure (3 atm) to ca. one-tenth volume and the volume reconstituted with water. This was repeated twice and about 6000 mL of filtrate (at least 60 volumes) collected altogether. The concentrate was freeze-dried and gave 1.3 g of plastein with a total nitrogen content of 13.5%.

Chemical Modification with the Purpose of Solubilizing Methionine-Enriched Plastein. *Oxidation with Dimethyl Sulfoxide Hydrochloric Acid (Fontana and Savige, 1977).* Fifty-five milligrams of plastein was dissolved in a mixture of dimethyl sulfoxide (80 μL) and glacial acetic acid (790 μL). The solution was kept at room temperature for 15 min and then diluted with distilled water. The reaction mixture was then evaporated under vacuum to dryness, redissolved in water, and evaporated again (twice). The product, dissolved in 1 mL of distilled water was then applied to a column of 1.6 × 50 cm of Sephadex G-10 in water. The peptide-containing fractions were pooled and freeze-dried to give 49 mg of product with total nitrogen content of 12.7%.

Oxidation with Hydrogen Peroxide at Alkaline pH. Fifty-four milligrams of plastein was suspended in 5 mL of 0.2 N NaOH. With gentle stirring at room temperature, 1.25 mL of 30% aqueous peroxide was added drop by drop over a period of 1.5 h. After 5 h the reaction mixture was neutralized with a few drops of 0.4 N HCl and the product applied to a column of Sephadex G-10 in water. Peptide-containing fractions were pooled and freeze-dried to give 43 mg of product with a total nitrogen content of 10.3%

Oxidation with Performic Acid. The same plastein was also subjected to performic acid oxidation according to standard procedures (Hirs, 1967).

Molecular Weight Estimation by Gel Filtration on Sephadex. Sephadex G-25, equilibrated in water, was poured into a column of 100 × 2.5 cm with a total volume of 400 mL. The void volume of the column was 180 mL. The column was eluted with distilled water at a flow rate of 18 mL/h.

Fifty milligrams of substrate peptides was applied together with 3 μCi of L-[^{14}C]methionine ethyl ester hydrochloride. In another run, 50 mg of each of the oxidized plasteins was likewise chromatographed. Two-hundred and fifty microliters of each fraction was incorporated into Aquasol liquid scintillant (15 mL) and tested for radioactivity in a Searle Mark III scintillation counter. Counting yields ranging between 90 and 92% were obtained. Fifty microliters of each fraction was hydrolyzed with 13 N NaOH by autoclaving and the hydrolyzate was subjected to the ninhydrin reaction (Hirs, 1967) in order to obtain quantitative information on the peptide content of each fraction. Methionine-rich plastein was analyzed in the same fashion by gel filtration on Sephadex G-25 in di-

Table I. Oxidation of Methionine-Enriched Plastein, Amino Acid Analysis

product	total N content, %	μg of Met equivalent, mg of product			Σμg/mg of product	Σμg/mg of N
		Met	sulfoxide	sulfone		
plastein	13.5	143.2			143.2	1060.7
plastein oxidized by Me_2SO/HCl	12.7	13.2	117.2		130.4	1026.8
plastein oxidized by H_2O_2/OH	10.3			111.0	111.0	1077.7
plastein oxidized by HCO_3H	10.7			119.1	119.1	1113.0

methyl sulfoxide as the solvent.

Molecular Weight Estimation by Determination of α-Amino Groups. We used the ninhydrin reagent described by Moore and Stein (1954) for this purpose. A calibration curve established with L-methionine showed strict linearity between quantity of methionine (range 0.02–1.00 μmol) and absorbance at 570 nm in a 1-cm path length cuvette (range 0.02–2.00 units).

Peptic digest of soy protein as well as oxidized, water-soluble plasteins (2.00 mg) were dissolved in 10 mL of 50% aqueous ethanol. One milliliter of this solution was mixed with another milliliter of ninhydrin reagent and incubated for 15 min at 100 °C in a boiling water bath. After cooling to 20 °C, the samples were measured in the spectrophotometer in 1-cm cuvettes at 570 nm. The measured absorbance was marked in the calibration curve and the methionine contents established from the graph. From this the content in α-amino nitrogen was calculated:

$$\mu\text{g of Met nitrogen} = \frac{(\mu\text{mol of Met}) \times (\text{mol wt of Met}) \times (\%\ \text{N Met})}{100} \quad (1)$$

The micrograms of methionine nitrogen are equivalent to the micrograms of α-amino nitrogen in the sample; therefore:

$$\%\ \alpha\text{-amino nitrogen} = \frac{(\mu\text{g of } \alpha\text{-amino nitrogen}) \times 100}{\mu\text{g of total nitrogen}} \quad (2)$$

Total nitrogen contents of the samples were determined on a Carlo Erba Model 1300 automatic nitrogen analyzer.

The average chain length ($\bar{n}$) was obtained by comparison of the α-amino nitrogen content of the samples with and without total acidic hydrolysis (6 N HCl, 24 h at 110 °C):

$$\frac{100}{\bar{n}} = \frac{(\%\ \alpha\text{-amino nitrogen product}) \times 100}{\%\ \alpha\text{-amino nitrogen hydrolyzed product}} \quad (3)$$

Amino Acid Analysis. Acid hydrolysis of plasteins followed by automatic amino acid analysis was performed according to standard procedures. Oxidized plasteins were, however, hydrolyzed in 4 N methanesulfonic acid (Pierce Chemicals, No. 25600) instead of 6 N hydrochloric acid in order to avoid reversion of methionine sulfoxide into methionine during hydrolysis.

RESULTS

Solubilization of Methionine-Enriched Plasteins. Soya-based plasteins could be enriched with methionine in a range varying from 5 to 40 wt % of incorporated methionine. When the plasteins contained at least 10% of covalently linked methionine, they became fully soluble in dimethyl sulfoxide. Plasteins with a lower content in methionine were only partially soluble in this solvent. The ease with which the plasteins were solubilized by hydrogen peroxide was, as well, clearly dependent on the methionine content of the product. A plastein with a very high content in methionine (>20%) seemed to us to be little representative of the plasteins which were described by Yamashita et al. (1971). For this reason, we prepared for our molecular weight study a plastein with approximately 15 wt % of methionine which was double the content in methionine of Yamashita's product, but fulfilled the conditions of solubility. According to our experience, 5 parts by weight of soy peptides and 1 part of ester hydrochloride had to be brought to reaction in order to achieve this. In fact, our product finally contained 14.32 ± 0.43 wt % of methionine. Using alkaline extraction followed by amino acid analysis of the extract without prior acidic hydrolysis, we showed that the product contained less than 0.5% of free methionine.

When the plastein was oxidized by the dimethyl sulfoxide procedure, essentially quantitative methionine recovery was obtained (Table I). Ninety percent of the total methionine was obtained as sulfoxide, 10% as methionine. This methionine might have arisen by partial reduction of the sulfoxide during hydrolysis in methanesulfonic acid.

Plastein oxidized with hydrogen peroxide at alkaline pH showed the same aminogram as the performic acid oxidized plastein: quantitative conversion of methionine into the sulfone (Table I).

Independently of the procedure of oxidation, the plastein derivatives were water-soluble at pH ≥ 5.0. Perfect solubility was observed at pH ≥5.5. At pH <5.0, however, they were gradually precipitated out of solution.

We considered the risk that the oxidation procedures might lead to cleavage of peptide bonds. For this reason, we subjected bovine β-lactoglobuline to both the dimethyl sulfoxide and the peroxide procedures. We analyzed the oxidized protein by gel filtration and electrophoresis and found it to be perfectly intact.

Average Chain Length of the Substrate Peptides. From the peptic digest of soy protein, a more homogeneous peptide fraction had been obtained by a two-step ultrafiltration procedure. The first step consisted of ultrafiltration on an Amicon UM-10 membrane (nominal cut-off = 10000 molecular weight), where the filtrate was collected. This filtrate was subjected to a second ultrafiltration with a UM-05 membrane (nominal cut-off = 500 molecular weight). This time the concentrate was saved. The peptides obtained by this procedure eluted as a broad peak with a few shoulders within the separation range of Sephadex G-25 (Figure 1). No peptide matter eluted in the excluded volume (molecular weight > 5000). In Figure 1 the elution profile of [^{14}C]methionine ethyl ester is also shown, clearly displaced from the peptide peaks. From the measurement of the α-amino nitrogen content of these peptides, which served as the substrate for plastein synthesis, a more precise value for their *average* molecular weight was obtained (Table II). The average chain length of the product was 5.5 residues, corresponding to an estimated average molecular weight of 700. Tsai et al. (1974) had found a very similar figure of 685 as the average molecular weight of peptic soy peptides.

Average Chain Length of the Plastein Peptides. Chromatography of ^{14}C-labeled plastein (methionine content 14.3%) on Sephadex G-25 in dimethyl sulfoxide essentially showed all the peptide matter to be eluted within

Table II. α-Amino Nitrogen Determinations of Substrate Peptides and Plasteins

product	total nitrogen content, %	(A) % α-amino N	(B) % α-amino N after complete hydrolysis	DH = (A × 100)/B	$\bar{n}$ = 100/DH
peptic digest (substrate peptides)	13.5	8.00[a]	43.85[a]	18.24	5.48
peptic digest (substrate peptides)	13.5	13.11[b]	78.67[b]	16.66	6.00
plastein (14.3 % methionine)	13.5	4-8[a,b]	74.20[a]		
plastein (14.3 % methionine)	13.5		68.30[b]		
plastein oxidized by Me_2SO/HCl	12.7	13.92[a]	79.90[a]	17.42	5.74
plastein oxidized by H_2O_2/OH	10.3	9.71[a]	59.80[a]	16.24	6.16

[a] Methionine used as a reference. [b] Leucine used as a reference.

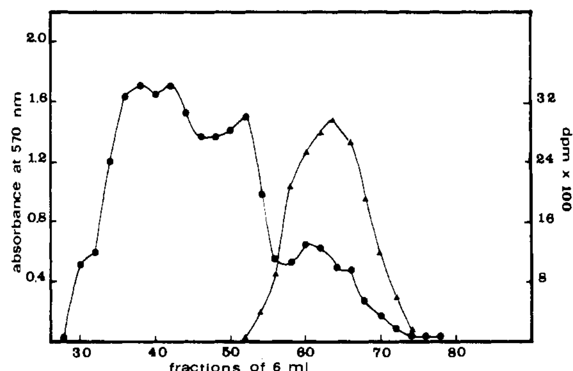

Figure 1. Elution profile of the substrate peptides for plastein synthesis on Sephadex G 25 in water. Fifty milligrams of peptic digest of soya protein dissolved in ca. 1 mL of water was applied to the column (100 × 2.5 cm) with a total volume of 400 mL and a void volume of 180 mL. The flow rate was 18 mL/h. The ninhydrin reaction was carried out with 200 μL of every second fraction after alkaline hydrolysis. (●) Absorbance at 570 nm; (▲) dpm/fraction of ^{14}C-labeled L-methionine ethyl ester.

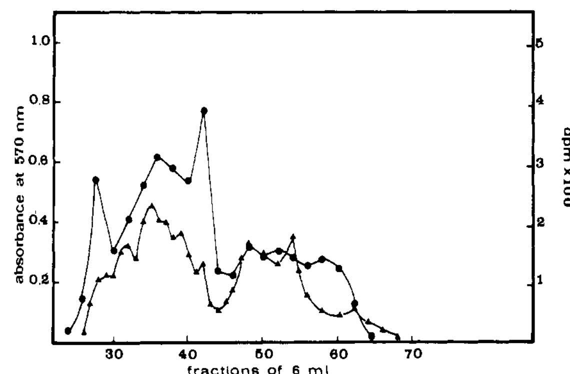

Figure 2. Elution profile of ^{14}C-labeled methionine–plastein on Sephadex G 25 in dimethyl sulfoxide. Fifty milligrams of plastein dissolved in 1 mL of dimethyl sulfoxide was applied on the column (100 × 2.5 cm) with a total volume of 330 mL and a void volume of 144 mL. The flow rate was 18 mL/h. The ninhydrin reaction was carried out with 150 μL of every second fraction. (●) Absorbance at 570 nm; (▲) dpm/fraction.

the separation range of the gel and none in the void volume. This indicated clearly that species of high molecular weight had not been produced during plastein synthesis. The elution profile of ninhydrin-reactive peptide matter and radioactivity were well congruent, indicating that a vast variety of the peptides had been labeled with methionine (Figure 2). The elution profiles of the water-soluble plastein derivatives brought no surprise as shown by the elution profile of peroxide-treated plastein from Sephadex G-25 (Figure 3). Again, a close similarity to the elution profile of the substrate peptides (also recorded in water) was evident. The absence of high-molecular-weight species which might have arisen by repeated peptide–peptide condensation was also evident with this product.

Chemical analysis of the α-amino nitrogen contents of oxidized, water-soluble plastein derivatives (Table II) gave an average 5.7 residue chain length for the plastein oxidized by Me_2SO/HCl and 6.2 residue in the sample treated with peroxide. We interpreted this to indicate an *average* chain length of six residues, the same as for the substrate peptides.

It is important to state here that α-amino nitrogen analysis performed on the insoluble plasteins gave entirely unreproducible results, suggesting in most cases higher chain length. This is apparently due to incomplete reaction of the N-terminals in the insoluble state.

DISCUSSION

Plastein synthesis occurring through peptide–peptide condensation must result in the formation of peptides which are in the average longer than the parent compounds. Such a plastein synthesis was described in the pepsin-catalyzed polymerization of oligopeptides isolated from peptone (Wieland et al., 1960).

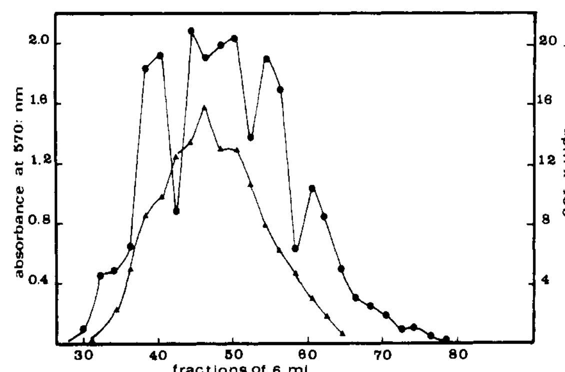

Figure 3. Elution profile of oxidized ^{14}C-labeled methionine–plastein on Sephadex G25 in water. Fifty milligrams of oxidized plastein dissolved in ca. 1 mL of water was applied to the column (100 × 2.5 cm) with a total volume of 400 mL and a void volume of 180 mL. The flow rate was 18 mL/h. The ninhydrin reaction was carried out with 200 μL of every second fraction after alkaline hydrolysis. (●) Absorbance at 570 nm; (▲) dpm/fraction.

Plastein synthesis might also be the result of transpeptidation (Horowitz and Haurowitz, 1959), in which case for each peptide bond which is formed another one was hydrolyzed before. In plastein synthesis with protein hydrolyzates as the substrate, it might be feasible that both transpeptidation and condensation reactions take place. As these substrates are usually complex mixtures of peptides with unknown structures, the study of the chemical mechanism of the reaction, e.g., by isotope techniques (Yamashita et al., 1974), may be difficult. Knowledge of the average chain length of the plastein peptides might inform us about the *predominant* mechanism of a given type of plastein synthesis as well.

Molecular weight estimates for plasteins published in the literature show a somehow confusing picture. Virtanen et al. (1950) found that, from a zein hydrolysate, pepsin produced plastein with molecular weight ranging between 2500 and 10000. These values were deduced from α-amino nitrogen measurements. These authors admitted, however, that according to cryoscopic methods, the molecular weight appeared to be only a few hundred daltons.

Tsai et al. (1974) deduced a molecular weight of approximately 20000 for a soy protein plastein by sedimentation analysis of the plastein in 6 M guanidium hydrochloride. Yamashita et al. (1970) claimed a threefold increase of the molecular weight of the peptides during chymotrypsin-catalyzed plastein synthesis. This result was obtained from α-amino nitrogen determinations and gel-filtration experiments. Furthermore, Yamashita et al. (1974) synthesized a glutamic acid rich plastein which was apparently marginally soluble in water. For this product they indicated a mean molecular weight of 12000, established by gel electrophoresis.

According to our experience, α-amino nitrogen determinations with insoluble plasteins gave unreproducible results, probably due to incomplete reaction of the N-terminals.

Moreover, we tested all the solvents and conditions described in the literature to dissolve plasteins and found none to achieve complete dissolution of a variety of plasteins. Dimethyl sulfoxide was the only solvent which dissolved many plasteins to an important extent and it dissolved completely plasteins which were enriched in methionine. We showed that methionine-rich plasteins could be converted to fully water-soluble products by partial or complete oxidation of the methionine. Careful analysis of these water-soluble plastein derivatives indicated that in our particular plastein, no increase in average chain length had occurred in the course of plastein synthesis. The synthetic process was clearly evidenced by the incorporation of radioactive methionine into the peptide bond. This result might be explained by transpeptidation reaction in the course of which a peptide substrate is first cleaved to give an activated peptidyl enzyme and a peptide fragment which is released. Aminolysis by methionine ester or another peptide would produce the new species.

We cannot rule out, however, that methionine might have been incorporated by condensation reactions into peptide bond, where it should then be mainly at the C-terminal. If peptide–peptide condensation occurred, the resulting products must have undergone again partial hydrolysis, as we were unable to find long chains in our product.

The insolubility of the plastein in water is not the result of formation of high-molecular-weight species but apparently the result of random rearrangement of water-soluble peptides into new water-insoluble species.

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