A Novel One-step Process for Enzymatic Incorporation of Amino Acids into Proteins: Papain-catalyzed Polymerization of L-Methionine Ethyl Ester and Its Regulation by Adding a Protein Substrate

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A one-step process has been developed as a novel modification of the plastein reaction, in order to incorporate L-methionine (ethyl ester form) directly into soy protein by treatment with papain.1,2) Our first report resulting from a series of this research has described the optimum conditions for the one-step process.1) The subsequent report has related a practical application of the process to commercial soy protein and flour for enhancing their methionine level in a large measure.2) In carrying out the process, however, it happens that L-methionine ethyl ester undergoes a kind of papain-catalyzed polymerization, particularly in the case of inadequate substrate (soy protein). This useless side-reaction is expected to be minimized if the methionine ethyl ester vs. soy protein concentration is well controlled. A main aim of the present paper is to define a critical point of concentration at which the methionine polymerization is regulated. In this context, some data are added with regard to a primary mechanism of the methionine incorporation into soy protein observed when the one-step process is carried out in the presence of adequate protein. Several abbreviations* are used in this paper.

MATERIALS AND METHODS

L-Methionine ethyl ester. A reagent grade preparation of L-methionine (Ajinomoto Co.) was esterified with ethanol by the method of Boissonas et al.3) The product was recrystallized from ethanol–ether–hexane (2:7:1) to obtain H-Met-OEt-HCl in needle. Checked by thin-layer chromatography,4) the compound gave a single spot with an Rf value of 0.65.

Substrate protein. A commercial preparation of soy protein isolate (Fuji Oil Mill Co.) was subjected to alkali and acid treatments as described in the preceding paper.2) A purified fraction resulting from isoelectric precipitation was used as substrate.

Enzyme. A papain preparation (Miles Laboratories, Inc.) was used. The activity for N-benzoyl-L-arginine p-nitroanilide was obtained as 3.24 units/mg.

* L-Methionine ethyl ester is abbreviated as H-Met-OEt. Methionine oligomers are described as H-Met2-OH, H-Met3-OH, etc. H-Metn-OH refers to a methionine polymer with a degree of polymerization of n. Concentrations of H-Met-OEt-HCl and soy protein in reaction system are written simply as [M] and [S], respectively. The concentrations are given in terms of weight percent unless otherwise noted. DNS, LAP and CP-Y are the abbreviations for 1-dimethylaminonaphthalene-5-sulfon, leucine aminopeptidase and carboxypeptidase Y, respectively.

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Incubation. Every experiment was carried out by using a 1 M carbonate buffer (pH 9) containing 40 mM L-cysteine. H·Met·OEt·HCl was dissolved in the buffer and incubated with papain in the presence or absence of the soy protein. Further details of the incubation conditions will be described later in respective cases.

Chromatography of methionine oligomers. An aliquot was sampled from each incubation mixture and treated with a three-fold volume of 1 N NaOH at 37°C for 1 hr to hydrolyze the ethyl ester linkage. The sample after the hydrolysis was quantitatively applied on a silica-gel thin-layer (Kieselgel 60 DC-Fertigplatten-Art. 5721, Merck), developed with butanol-acetic acid-water (4: 1: 2), and sprayed with a usual ninhydrin reagent. H·(Met)₂·OH and H·(Met)₃·OH were identified by the DNS method.

Quantification of methionine polymer. An insoluble methionine polymer, H·(Met)ₙ·OH, was precipitated by centrifuging an alkali-treated incubation mixture at 10,000 rpm for 30 min, washed out with sufficient water, and weighted after dehydration.

Determination of average degree of polymerization. H·(Met)ₙ·OH was treated with DNS chloride by the usual method. The DNS derivative was hydrolyzed with 6 N HCl in a deairated tube at 105°C for 48 hr. The term n was obtained by comparing the molar amount of DNS-methionine and that of the free methionine produced by the acid hydrolysis.

Determination of methionine incorporated into protein. A sample resulting from the incubation of H·Met·OEt·HCl with papain in the presence of soy protein was alkali-treated and analyzed for free methionine as mentioned in the preceding paper. The degree of incorporation was obtained by subtracting the determined value of the free methionine from the original H·Met·OEt·HCl concentration (IM) on a molar basis.

Degradation of a methionine-incorporated protein with exopeptidases. A part of the sample was treated with LAP under the conditions proposed by Hill and Smith. A time-course liberation of amino acids during the treatment was followed. On the other hand, another part of the same sample was treated with CP-Y according to the method of Hayashi et al. The amino acid liberation was followed likewise.

RESULTS

Papain-catalyzed polymerization of L-methionine ethyl ester

The process was carried out in the absence of soy protein as follows. H·Met·OEt·HCl (10 g) was dissolved in 1 M NaHCO₃ (50 ml) containing 40 mM L-cysteine and the solution adjusted to pH 9 by adding a small amount of diluted NaOH. A total volume of the solution was adjusted finally to 100 ml. Papain (2 g) was added and the mixture incubated at 37°C. Aliquots were pipetted at appropriate times during the incubation, treated with alkali to hydrolyze the ethyl ester linkage, and submitted to thin-layer chromatography. Figure 1 shows the chromatogram of separated H·Met·OH, H·(Met)₂·OH and H·(Met)₃·OH. Besides those, an insoluble substance occurred which remained at the original point on the thin-layer plate. It was observed that the substance appeared at 1 hr from the initiation of the incubation, with a gradual increase in quantity thereafter. The analysis by the DNS method indicated that the insoluble substance was a methionine polymer having an average degree of polymerization of 7.4. No other ninhydrin-positive spots were found on the chromatogram.

Time-course changes in quantity of H·Met·OH, H·(Met)₂·OH, H·(Met)₃·OH and H·(Met)ₙ·OH (n=7.4) were investigated. H·
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TABLE I. TIME-COURSE FORMATION OF FREE METHIONINE, DIMETHIONINE, TRIMETHIONINE AND METHIONINE POLYMER FROM METHIONINE ETHYL ESTER DURING INCUBATION WITH PAPAIN

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Substrate</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-Met-OEt (wt%)</td>
<td>H-Met-OH (wt%)</td>
</tr>
<tr>
<td>0 sec</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1 min</td>
<td>98.0</td>
<td>n.d. b</td>
</tr>
<tr>
<td>5</td>
<td>98.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>10</td>
<td>97.9</td>
<td>1.2</td>
</tr>
<tr>
<td>30</td>
<td>96.0</td>
<td>—</td>
</tr>
<tr>
<td>1 hr</td>
<td>89.8</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>51.7</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>24.3</td>
<td>6.0</td>
</tr>
<tr>
<td>12</td>
<td>17.2</td>
<td>—</td>
</tr>
<tr>
<td>1 day</td>
<td>15.8</td>
<td>6.7</td>
</tr>
<tr>
<td>2</td>
<td>15.0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

a \(n=7.4\).

b Not detectable.

Met·OH was determined directly with an amino acid autoanalyzer. H·(Met)₂·OH and H·(Met)₃·OH were extracted from the thin-layer plate, hydrolyzed with 6 N HCl at 110°C for 24 hr, and quantified with the autoanalyzer. H·(Met)₄·OH was weighed in the way described already. The resulting data are summarized in Table I.

An experiment was conducted to relate the yield of H·(Met)₄·OH with the initial concentration of H·Met·OEt·HCl ([M]). No insoluble substance was apparently yielded at [M]=1 (wt %) or lower. H·(Met)₄·OH was formed in a small yield at [M]=1.5, with an appreciable increase at [M]=2 or higher.

Figure 2 illustrates the increasing yield as a function of [M].

Papain-catalyzed incorporation of L-methionine ethyl ester

An investigation was made to confirm whether the papain-catalyzed polymerization of H·Met·OEt is effectively regulated by adding a protein substrate to the reaction system. H·Met·OEt·HCl was dissolved in the carbonate buffer so as to be a concentration ([M]) ranging from 0.1 to 5.0 wt% . To the solution was added the soy protein at a concentration ([S]) of 1, 5, 10 or 20 wt%. The incubation was carried out in the presence of papain (0.2 wt% of the incubation mixture) at 37°C for 24 hr. An entire reaction product after the incubation was centrifuged to remove the supernatant and the precipitate quantified as mentioned before. Table II shows the result, indicating that the polymer formation highly depends on [S]. More exactly, a lower [M] as well as a higher [S] are effective in regulating the self-polymerization of methionine. The term, [M]/[S], can thus be an important factor for the regulation. With regard to [M]/[S] there was a critical point in each case of combination of [M] and [S] (Table II).

A degradation experiment with LAP and
TABLE II. CONDITION FOR PAPAIN-CATALYZED POLYMERIZATION OF METHIONINE ETHYL ESTER AND ITS REGULATION, WITH A CRITICAL LINE ABOVE WHICH THE POLYMERIZATION IS COMPLETELY REGULATED\(^a\)

<table>
<thead>
<tr>
<th>[M](^b)</th>
<th>[S](^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2.0</td>
<td>5.2</td>
</tr>
<tr>
<td>2.5</td>
<td>49.3</td>
</tr>
<tr>
<td>5.0</td>
<td>56.2</td>
</tr>
</tbody>
</table>

\(^a\) Each value is given by weight percent. The value of 100 is reached when the substrate, H\(\cdot\)Met\(\cdot\)OEt, is completely polymerized.

\(^b\) Concentration of H\(\cdot\)Met\(\cdot\)OEt\(\cdot\)HCl (wt%) in the reaction system.

\(^c\) Concentration of the soy protein (wt%) in the reaction system.

CP–Y was conducted. The methionine-incorporated sample to be subjected to the degradation was prepared under the condition, [M]=0.1 and [S]=1 (wt%). Papain was used in a proportion of 1% of the soy protein. The incubation was carried out at 37°C for the short period of 2 hr, because a longer time of incubation possibly caused to give a complicated product. The entire reaction product resulting from the incubation under the abovementioned condition was alkali-treated and dialyzed with a cellophane tube in running water at 5°C for 2 days. Since no free amino acids were detected in the non-diffusible fraction, this was freeze-dried to a powder. Its yield was 39.4 g from 100 g soy protein on a dry-matter basis. The methionine content in this sample was found to be 2.27% of its total protein as measured based on N\(\times\)6.25. An aliquot of the sample was treated with LAP and another aliquot with CP–Y. In the former case it was observed that methionine was liberated very slowly compared to any other amino acid, whereas in the latter the liberation rate of methionine was much greater than those of most other amino acids (Fig. 3).

FIG. 3. Liberation of Amino Acids during Treatment of a Methionine-incorporated Product with Leucine Aminopeptidase (LAP)\(^a\) and with Carboxypeptidase Y (CP–Y)\(^b\).

\(^a\) Identity: 1, Ser plus Asn; 2, Phe; 3, Glu; 4, Thr plus Gln; 5, Leu; 6, Ile; 7, Tyr; 8, Ala; 9, Asp; 10, Gly; 11, Val; 12, Arg; 13, Lys.

\(^b\) Identity: 1, Leu; 2, Ile; 4, Phe; 5, Val; 6, Ser plus Asn; 7, Ala; 8, Tyr; 9, Thr plus Gln; 10, Glu; 11, Gly; 12, Arg; 13, Lys; 14, Asp.

DISCUSSION

Earlier studies have disclosed that amino acids and their active derivatives are polymerized by the action of proteases through, for example, condensation, transamidation and transesterification; a detailed review has been made by Fruton.\(^9\) More recently, Sluyterman and Wijdenes\(^10\) have found that the catalytic action of papain produces insoluble polyleucine peptides, 8–9 units long, from L-leucine methyl ester. They have analyzed the mechanism of the polymer formation and concluded that the polymerization reaction proceeds by the combination of a very slow dimerization rate and a high rate of chain growth from the trimer onwards, until the insoluble peptide mixture precipitates as the final product. This phenomenon seems to be quite similar to that observed...
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SCHEME I.

By analogy of the leucine polymerization\(^{(10)}\) as well as from our results shown in Table I and Figure 2, it is speculated that the papain-catalyzed polymerization of H·Met·OEt proceeds as illustrated above (Scheme I). The methionyl-papain intermediate, as referred to as H·Met·O-[papain], must be formed as the first stable covalent product, which can be hydrolyzed to free methionine on one hand (Fig. 1). On the other, the intermediate can undergo the aminolysis by another molecule of H·Met·OEt as a nucleophile amine, with formation of the dimer, H·(Met)\(_2\)·OEt. This can act as an acylation reagent to form the next intermediate, H·(Met)\(_2\)·O-[papain], which in turn is aminolyzed by H·Met·OEt to the trimer. The subsequent process may proceed faster to accumulate the insoluble polymer, H·(Met)\(_n\)·OEt (n=7.4), in the long run.

When the incubation is carried out in the presence of soy protein, small peptides must occur as the hydrolysis products. If one of these peptide acts as a nucleophile amine and attack any particular one of the acyl-papain intermediates including H·Met·O-[papain], a product can be formed in which the incorporated methionine residue is located at the N-terminal. The occurrence of such a process (Scheme II) may not be highly possible, since the rate of liberation of methionine from the sample during treatment with LAP is extremely slow (Fig. 3).

SCHEME II.

In contrast, Scheme III would be able to explain a highly possible process taking place during the incubation in the presence of adequate soy protein. It may be much easier for papain to interact first with a protein molecule with formation of the peptidyl-papain intermediate. The aminolysis of this intermediate

SCHEME III.

REFERENCES