Papain-Catalyzed Hydrolysis of and Amino Acid Incorporation into BSA and Zein Substrates in Low Water Organic Media

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Papain, either modified with polyethylene glycol (PEG) or in the form of a suspended powder, was used to catalyze the digestion of protein substrates in organic solvents to gain insights into protein–protein interactions in media of this kind. Bovine serum albumin was efficiently hydrolyzed in toluene containing 1–2% added water (v/v). The extent of proteolysis, as estimated by amino group determination, depended on the water concentration and, under the optimal condition, was 20% higher than that obtained in aqueous media using the same enzyme/substrate ratio. The highest rate of hydrolysis was obtained in the most hydrophobic solvent, but the attachment of polyethylene glycol chains to the enzyme did not enhance the catalysis. Proteolysis in organic solvents can be said to constitute an interesting approach to the enzymatic digestion of proteins and amino acid incorporation involved in aminolysis. In the case of the hydrophobic protein zein, more than 10% of the peptide bonds were hydrolyzed by PEG–papain in toluene, and in the presence of a potent nucleophile such as Lys-OMe or Lys-NH_2 the transpeptidation reaction resulted in an increase in the lysine content of zein from 0.15% to 3.5% and 10.4%, respectively. The use of organic media therefore provides a potentially useful approach to the enzymatic modification of food protein.

INTRODUCTION

It has by now been clearly established that proteases are active in organic media, where they catalyze ester synthesis, ester exchange, or aminolysis by synthesizing new peptide bonds rather than by hydrolysis. The properties of hydrolytic enzymes in these media have been extensively investigated using the biocatalyst either in the form of a dry powder (Klibanov, 1986) or immobilized on an inert support (Reslow et al., 1987) or modified with polyethylene glycol (PEG) to make the enzymes soluble in organic solvents (Oka and Morihara, 1978; Matsushima et al., 1984). Regarding protease-catalyzed peptide synthesis, high reaction yields have been obtained with PEG-modified chymotrypsin (Gaertner and Puigserver, 1988), thermolysin (Ferjancic et al., 1988), and papain (Gaertner et al., 1990) in benzene or 1,1,1-trichloroethane. On the other hand, subtilisin exhibited a high propensity for catalyzing transesterification reactions, contrary to aminolysis in benzene (Ferjancic et al., 1990). These results, like those published on other esterases (Cesti et al., 1985), were obtained with substrates having a low molecular mass that were soluble or partially soluble in organic solvents. The question therefore arose as to whether catalysis with water-soluble substrates such as proteins may also take place in organic solvents. We recently observed that trypsin, either modified with polyethylene glycol or in the form of a suspended powder in organic solvents, was able to catalyze the digestion of protein substrates (Gaertner and Puigserver, 1991). These results are of interest and broaden the scope of studies on biocatalysis in these media, since a large variety of enzymes function with water-soluble substrates, and in many cases water participates directly in the reaction. Though little transpeptidation reaction occurred with PEG–trypsin, it is likely that catalysis in organic media will promote aminolysis over hydrolysis, contrary to what occurs in the case of the reaction in all-water media.

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The presence of a hydrophilic polypeptide substrate able to adsorb high amounts of water will modify the partitioning of water between the biocatalyst and the bulk phase. While enzymatic peptide synthesis with amino acid derivatives has occurred in organic media containing less than 0.1% water (Gaertner et al., 1990), higher concentrations of water will be necessary in this case to express catalytic activity, which depends strongly on the amount of water entrapped around the enzyme.

Since papain has already been found to catalyze the polymerization of amino acid derivatives (Anderson and Luisi, 1979) and their incorporation into protein in the aqueous phase (Yamashita et al., 1979a,b; Monti and Jost, 1979), it seemed to be of interest to investigate the catalytic properties of the enzyme in organic solvents, in the presence of a protein substrate.

The purpose of this study was to investigate whether papain, either in the form of a solid enzyme powder or modified with polyethylene glycol, can be used in low water organic media to incorporate amino acid derivatives efficiently into proteins. This is a new approach to the enzymatic modification of food proteins for nutritional and functional improvement.

MATERIALS AND METHODS

Materials. Papain (EC 3.4.22.2) was purchased from Boehringer (Mannheim, FRG) as a crystalline suspension in 0.05 M sodium acetate–0.2 M sodium chloride (pH 4.5). Monomethoxypolyethylene glycol (PEG, M_r 5000 and 2000), cyanuric chloride, *p*-nitrophenyl chloroformate, phenylalanine oligomers up to five amino acid residues, bovine serum albumin, and zein from corn were supplied by Sigma Chemical Co. (St. Louis, MO). The mercaptoethanol used as papain activator was obtained from Fluka AG (Buchs, Switzerland) as were the *N*-ethyldiisopropylamine (DIPEA) and L-methionine ethyl ester hydrochloride (Met-OEt·HCl). L-Methionine amide hydrochloride (Met-NH_2·HCl) was provided by AEC/Rhône-Poulenc (Commentry, France), while di- and trimethionine, L-lysine amide dihydrochloride (Lys-NH_2·2HCl), L-lysine methyl ester hydrochloride (Lys-OMe·2HCl), lysine oligomers from two to five amino acid residues, and L-phenylalanine amide (Phe-NH_2) were from Bachem (Bubendorf, Switzerland). Tetramethionine and pen-

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tamethionine were synthesized as previously reported (Gaertner and Puigserver, 1984). Anhydrous solvents containing less than 100 ppm of water were supplied by SDS (Peypin, France) and were further dried by storage over a 0.4-nm molecular sieve. All other commercial reagents and chemicals were of analytical grade.

Preparation of Polyethylene Glycol Modified and Lyophilized Papain. Modification of papain with PEG_{2000} and PEG_{5000} *p*-nitrophenyl carbonate was carried out as previously described (Gaertner and Puigserver, 1991). The enzyme was dissolved in a sodium tetraborate buffer (0.1 M, pH 8.5, 2 mg/mL). After adding a 10 M excess of activated polyethylene glycol over the amino groups, the resulting mixtures were gently stirred at 20 °C for 4 h and successively dialyzed at 4 °C against sodium acetate buffer (50 mM, pH 5.5) and against water, using an ultrafiltration cell fitted with an Amicon YM 10 or PM 30 membrane (cutoff M_r 10 000 and 30 000) for PEG_{2000}– and PEG_{5000}–papain, respectively, and finally lyophilized.

Papain was also modified with cyanuric chloride activated methoxypolyethylene glycol with a M_r of 5000 ($PEG_{2\times5000}$–papain) as previously described (Gaertner and Puigserver, 1989). The papain used in the form of suspended powder was obtained by lyophilizing an enzyme solution (10 mg/mL) which had been dialyzed against a 50 mM sodium phosphate buffer, pH 6, overnight at 4 °C. The amidase activity of modified papain was determined spectrophotometrically using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPA) as a substrate (Arnon, 1970). The activities in water of PEG_{2000}–, PEG_{5000}–, and PEG_{10000}–papain and lyophilized papain were 84%, 90%, 70%, and 80%, respectively, with respect to the activity of untreated papain.

Protein Substrate. Commercial, crystallized bovine serum albumin, containing 96–99% albumin (w/w), was dialyzed against water and freeze-dried. Zein from corn was solubilized in an ethanol–water mixture (70:30 v/v) and dialyzed against water at 4 °C. The precipitate was separated by centrifugation, freeze-dried, and used as a protein substrate without further purification.

Proteolysis in Organic Solvents. The standard reaction mixture in organic solvent (0.5 mL) consisted of 1 mg of protein, 1–6% water (v/v) depending on the solvent used, 0.2% mercaptoethanol (v/v), and 17 μM modified papain. When zein was used, 8–10% ethanol (v/v) was required to solubilize the protein substrate in the water-immiscible organic solvent. Reactions were performed at 37 °C for 17 h with continuous magnetic stirring and stopped by adding 30 μL of a 0.1 M iodoacetic acid solution in ethanol. The mixture was then evaporated to dryness under reduced pressure, and the dried material dissolved in an aqueous solution for analysis: 0.4 mL of sodium hydroxide (0.12 N) for BSA or 0.4 mL of a 0.2 M sodium phosphate buffer, pH 7.0, containing 1% SDS (w/v) and 10% ethanol (v/v) for zein. Under these conditions, the peptides were completely solubilized and papain was totally inactivated since no proteolysis at all could be detected in the aqueous solution. The protein hydrolysis was determined by measuring the release of amino groups by the 2,4,6-trinitrobenzenesulfonic acid method (Fields, 1972). In addition, peptide digests of BSA and zein were characterized by polyacrylamide gel electrophoresis (PAGE) using the methods by Laemmli (1970) and Swank and Munkrees (1971), respectively. In the latter case, the gel was stained with silver nitrate.

Enzymatic Incorporation of Amino Acids into Proteins. The reaction conditions were the same as those described above in the case of proteolysis, except that an amino acid derivative was added to the reaction mixture (final concentration 10 mM) in the presence of 0.8% DIPEA to solubilize the nucleophile in the organic solvent.

Determination of Amino Acid Incorporation. The extent of amino acid incorporation was estimated either by measuring the free unreacted amino acid content in the reaction mixture or from the amino acid composition of the refined product. In the first case, an aliquot of the water-resolubilized reaction mixture was directly applied to the amino acid analyzer (Beckman apparatus system 6300 HP) to determine the free amino acid content. Since a comparable determination was performed in control experiments without any enzyme, the percentage incorporation of amino acid could be calculated. In the second case, the protein fraction was first separated from the free amino acid derivatives by either dialysis against water or precipitation with anhydrous acetone (4 v/v) at −20 °C for 15 min. In the former case, the dried reaction mixture was resolubilized in 0.1 N NaOH to completely hydrolyze the remaining amino acid ester to free amino acid, then extensively dialyzed against water in a wet cellulose dialysis tube (cutoff M_r 1000), and finally freeze-dried. In the latter case, the dried material was resuspended in methanol, sonicated, and centrifuged. The washing of the protein fraction was repeated three times, and the resulting material was dried under vacuum. Control experiments showed that the resulting protein fractions did not contain any free amino acid. The refined products were hydrolyzed in 5.6 N HCl at 110 °C for 24 h and then analyzed to determine the amino acid composition. The results were compared with those obtained in control experiments carried out with protein substrate and enzyme but no amino acid derivative.

Papain-Catalyzed Amino Acid Polymerization in Organic Solvent. The reaction conditions were comparable to those described above and carried out either in toluene or in ethyl acetate containing 1% and 6% water (v/v), respectively, but in the absence of protein substrate. The concentration of the monomer, i.e., amino acid ester or amide, was usually 10 mM. The reaction was stopped by adding 100 μL of ice-cold acetic acid and then dried under reduced pressure. The average degree of polymerization was estimated by HPLC. The samples were redissolved in 4% (v/v) formic acid and analyzed on a Waters system fitted with an Ultrabase C_8 reversed-phase column (Société Française de Chromatographie sur Colonne, 5 μm, 4.6 × 250 mm) using a flow rate of 1 mL/min and a linear gradient of acetonitrile in 0.05% TFA. The effluent was monitored at 214 nm. The reaction products were identified by means of synthetic oligomers with up to five amino acid residues. As previously established in the case of other amino acid oligomers (Sasagawa et al., 1982), the retention time of mono-, di-, tri-, tetra-, and pentamethionine obeyed a logarithmic regression, depending on the degree of polymerization, so that it was possible to determine the degree of polymerization of oligomers from their elution time on the chromatogram. These results were further confirmed by mass spectrometry. Unknown peaks were collected and samples were either hydrolyzed in 5.6 N HCl at 110 °C for 24 h for amino acid analysis or characterized by fast atom bombardment mass spectrometry (FAB-MS) using a Nermag R-30-10 equipment as previously reported (Barber et al., 1982). For FAB-MS analysis in the positive ion mode, the stainless steel target was loaded with 1 μL of a mixture of glycerol and 1-thioglycerol (50:50 v/v). Samples were dried and taken up in methanol. One microliter was then applied to the loaded target.

RESULTS AND DISCUSSION

Proteolytic Activity of Papain in Organic Solvents. Effects of the Water Concentration. The proteolytic activity of PEG_{2000}–papain on BSA in toluene was estimated by determining the amino groups of the released peptides which were characterized by polyacrylamide gel electrophoresis (PAGE) (results not shown). Peptides in the 10 000–50 000 range were detected. As shown in Figure 1, no hydrolysis was found to occur with less than 0.5% water (v/v) in the reaction medium. On the other hand, beyond this value (0.6–0.8% v/v), the higher the water content in the reaction mixture, the higher the proteolysis. A plateau was observed at a water concentration range of 1.5–4% (v/v), but at the highest concentrations, the reaction mixture was comparable to a two-phase system. Control experiments without enzyme or protein substrate showed that no significant protein or enzyme autolysis occurred under the reaction conditions used. In a previous work, the partition of enzymatic activity between the organic solvent-soluble phase and the protein substrate fraction in the reaction mixture as influenced by water concentration has been investigated (Gaertner and Puigserver, 1991). Almost all of the activity was found to be shifted from the hydrophobic solvent to the protein substrate insoluble fraction when the water content exceeded 0.6%. In fact, the adsorption of the soluble enzyme at the protein–organic solvent interface is a prerequisite for catalysis. Consequently, the proteolytic

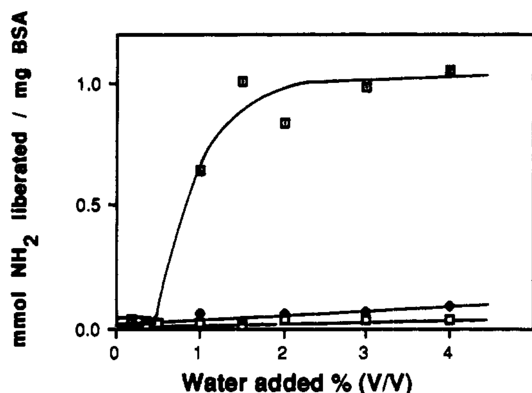

Figure 1. Extent of BSA digestion by PEG_{2000}–papain in toluene after 17-h incubation at 37 °C as a function of the amount of added water. The reaction mixture consisted of 0.2% mercaptoethanol (v/v) and 17 μM PEG_{2000}–papain in 0.5 mL of toluene containing 0–20 μL of added water, with (⊡) and without (□) 1 mg of BSA. A control experiment was performed without any enzyme (●).

activity could only be detected when the amount of water in the reaction medium was increased to 0.6–0.8% (v/v).

The approximately 10-fold higher concentration of water necessary to induce catalytic activity in PEG–papain in the presence of a protein substrate, as compared to enzyme-catalyzed peptide synthesis (Gaertner et al., 1990), may be due to the ability of the hydrophilic substrate to adsorb large amounts of water when suspended in a water-immiscible organic solvent. When a highly hydrophilic protein substrate is added to an enzyme-containing organic medium, the substrate may act the part of a support material for the enzyme and therefore have a strong disturbing effect on water distribution between the enzyme and the hydrophobic organic medium. In toluene, more than 0.5% added water (v/v) was required to hydratize both the protein substrate and the enzyme and give rise to proteolytic activity. Moreover, the organic solvent is also known to interact directly with the necessary water in the vicinity of the enzyme and, depending on its polarity, to be able to solubilize large amounts of water. As shown in Figure 2A, comparable rates of hydrolysis were obtained in the most hydrophobic solvents, benzene and toluene, while the hydrolytic activity was significantly lower in 1,1,1-trichloroethane in the presence of the same amount of added water, 1% (v/v). Although the extent of the digestion appears to have been more pronounced in water after a 17-h incubation period than in toluene, it is important to remember that proteolysis in organic media depends on the water concentration and that still higher values of released amino groups could be obtained in toluene containing 1.5% water (v/v) (Figure 1), which were 20% higher than those obtained in the 5 mM sodium phosphate buffer at pH 7. Whatever the solvent used, PEG_{2000}–papain retained more than 90% of its initial activity after a 90-min incubation period (as determined by estimating the residual enzyme activity in water after the organic reaction mixture was dried under reduced pressure), and approximately the same degree of hydrolysis was still observed after a 17-h incubation period. In most hydrophobic solvents, a 1% water concentration seems to suffice to hydrate both the protein substrate and the enzyme and thus to induce catalytic activity. As was expected, the length of the polyethylene glycol chains attached to the amino groups of the enzyme also influenced the enzyme–protein interactions (Figure 2B). Since the same amount of enzyme with respect to the activity levels in water was used in all of the experiments, the catalytic activity of the various derivatives in benzene could be compared and was as follows: powdered papain > PEG_{2000}–papain > $PEG_{2\times5000}$–papain ≥ PEG_{5000}–papain. The loss of activity during a 2-h incubation period was about 30% with all of the papain derivatives. These results confirm, on the one hand, that steric hindrance of PEG chains considerably reduces the activity of modified papain toward protein substrates and, on the other hand, that the stability of the enzyme does not increase with the size of the PEG moiety.

Enzymatic Incorporation of Amino Acids into BSA. The reaction conditions described above were used to investigate the ability of the enzyme to incorporate amino acid derivatives into BSA. Since it was observed that in the presence of a polypeptide substrate more than 90% of the water added was adsorbed by the protein, it can be assumed that when the protein concentration is varied in the reaction mixture, the protein/water ratio has to be kept constant to sustain the activity of the enzyme catalyst. In fact, when phenylalanine amide was used as substrate, no significant changes in the phenylalanine incorporation rate (Phe/Phe_0) in the final product were observed when the protein concentrations were varied from 2 to 4 mg/mL, while the water/protein ratio was maintained constant (5 μL/mg of protein) (Table I). Using a 20 mM $Phe\text{-}NH_2$ concentration in the reaction medium, an approximately 4.0-fold increase in the Phe content of BSA was obtained, but as expected, the higher the protein concentration, the lower the amount of remaining free $Phe\text{-}NH_2$. Increasing the amino acid concentration did not increase the extent of the phenylalanine incorporation into BSA (results not shown). In this process, a polymerization reaction of the

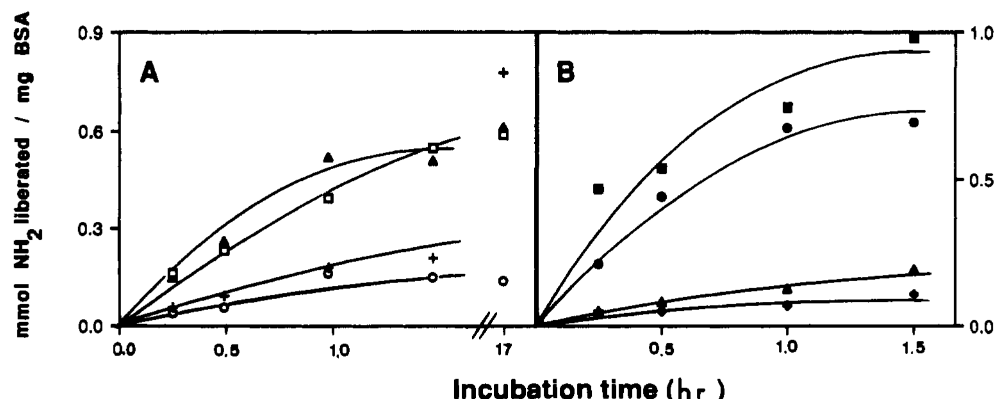

Figure 2. Influence of the medium (A) and of the type of catalyst (B) on the time course of the digestion of BSA in the presence of 1% added water (v/v). The reaction conditions were the same as in Figure 1 except that benzene (□), toluene (Δ), 1,1,1-trichloroethane (O), or an aqueous sodium phosphate buffer (5 mM, pH 7) (+) was used to solubilize the initial material (A). Reactions with PEG_{2000}– (●), PEG_{5000}– (◆), $PEG_{2\times5000}$– (▲) papain, or papain in the form of a suspended powder (■) were performed in benzene (B).

Table I. Enzymatic Incorporation of L-Phenylalanine Amide into BSA as a Function of Protein Concentration in the Reaction Medium[a]

BSA concn, mg/mL	(Phe/Phe$_0$)[b]	remaining free Phe-NH$_2$[c] %
2	3.80 ± 0.1	42.0 ± 6.7
3	3.65 ± 0.3	28.8 ± 0.9
4	4.20 ± 0.2	13.7 ± 5.0

[a] The reaction mixture (0.5 mL) in toluene consisted of 1.0, 1.5, or 2.0 mg of BSA, 20 mM Phe-NH$_2$, 0.8% DIPEA (v/v), 0.4% MeOH (v/v), 0.2% mercaptoethanol (v/v), and 17 μM PEG$_{2000}$–papain with the added water/protein ratio maintained constant (5 μL of water/mg of protein) and was incubated at 37 °C for 17 h. [b] The Phe/Phe$_0$ ratio corresponds to the ratio between the amino acid content of product refined by extraction and the amino acid content of the control preparation in which protein substrate was incubated with the amino acid amide, in the absence of the enzyme. [c] The free Phe-NH$_2$ was directly estimated by performing amino acid analysis on the crude reaction mixture.

Table II. PEG$_{2000}$–Papain-Catalyzed Incorporation of Various Amino Acid Derivatives into BSA in Benzene[a]

amino acid incorporated	remaining free amino acid derivative, %	amino acid content,[b] wt %: native protein hydrolysate	product refined by extraction	product refined by dialysis
H-L-Lys-NH$_2$·2HCL*	5.00	8.59	12.00	16.40
H-L-Lys-OMe·2HCL*	42.20	8.61	14.13	13.55
H-L-Met-NH$_2$·HCL*	0.00	0.79	26.61	28.88
H-D-Met-NH$_2$·HCL*	88.50	0.81	0.99	2.17
H-L-Met-OEt·HCL	43.00	0.87	15.03	15.02
H-L-Phe-NH$_2$	23.90	4.98	14.77	27.73
H-L-Phe-NH$_2$*	19.80	5.19	15.13	18.96

[a] 1 mg of BSA, suspended in 0.5 mL of benzene containing 17 μM enzyme, 0.8% DIPEA (v/v), 0.2% mercaptoethanol (v/v), 1% water (v/v), and 10 mM free amino acid derivative, was incubated for 17 h at 37 °C. Stock solution of the free amino acid derivative was prepared either in the added water fraction (*) or in benzene containing 2% DIPEA and 1% EtOH. [b] Amino acid content of the native protein and the refined product with respect to the amino acid used in the incorporation reaction.

amino acid derivative involving no incorporation into the protein might be expected, as already occurs in water (Arai et al., 1979). When the reaction was carried out in the absence of protein substrate, but under the same conditions of hydratation, 30% of the initial Phe-NH$_2$ disappeared from the reaction mixture, forming phenylalanine oligomers as assessed by HPLC (results not shown). Since the enzymatic reaction in the presence of high protein concentrations involved the consumption of up to 85% of the initial amino acid substrate, it can be assumed that incorporation is the main reaction catalyzed by papain under these conditions.

Various amino acid derivatives were investigated as possible nucleophile acceptors in papain-catalyzed BSA digestion in benzene (Table II). The incorporation efficiency was estimated by determining either the remaining free amino acid in the reaction mixture or the amino acid composition of the refined product after washing with methanol or dialysis against water. In either case, extraction or dialysis, the yields of plastein recoveries ranged from 80% to 100%. As previously observed in enzymatic peptide synthesis, amide derivatives are more efficient acceptor nucleophiles for the acyl–enzyme intermediate than ester derivatives. An almost quantitative reaction was obtained with L-lysine amide and L-methionine amide. The low lysine content of Lys–plastein as compared to the almost total consumption of the amino acid amide may be attributable, however, to the occurrence of a considerable polymerization side reaction involving no effective incorporation into the plastein. Moreover, the occurrence of this reaction in the case of hydrophobic amino acids (Met, Phe derivatives) helps to explain the

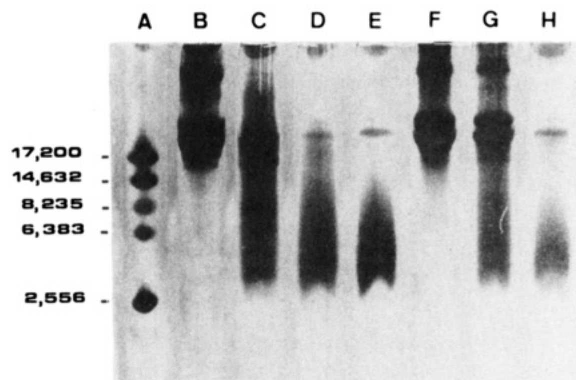

Figure 3. SDS–PAGE profiles in 8 M urea of zein digests obtained with PEG$_{2000}$–papain after various incubation periods in toluene and ethyl acetate. (Lane A) Molecular weight markers; (lanes B–E) proteolysis in toluene, in the presence of 1% added water (v/v), 0.2% mercaptoethanol (v/v), 8% ethanol (v/v), after a 0-, 0.5-, 1.0-, and 2.5-h incubation period, respectively; (lanes F–H) digests obtained after 0-, 4-, and 20-h incubation in ethyl acetate containing 6% water (v/v), 8% ethanol (v/v), and 0.2% mercaptoethanol (v/v).

discrepancies between the amino acid contents of the refined products depending on the method of purification used. Higher values obtained in the case of amino acid–plasteins refined by dialysis may be attributable to the presence of some oligomerized insoluble material which is difficult to separate by this process. The polymerization side reaction is probably regulated by the protein/amino acid derivative ratio as in the case of the plastein reaction described in water (Arai et al., 1979).

Hydrolysis of Zein and Amino Acid Incorporation. Hydrolysis and amino acid incorporation experiments were extended to zein, a water-insoluble protein, which can be said to be a model protein for experiments in nonaqueous media. Zein was solubilized in toluene or ethyl acetate, in the presence of 8% and 12% (v/v) ethanol, respectively. The PEG$_{2000}$–papain-catalyzed hydrolysis of zein (0.2% w/v) was dependent as in the previous cases on the water content of the reaction mixture. The extent of protein digestion in toluene reached a plateau after a 2-h incubation period and also depended on the water concentration (0.5–2% v/v). The higher extent of proteolysis (0.7 mmol of NH$_2$ liberated/mg of zein) was obtained in toluene containing 2% (v/v) added water. Urea–polyacrylamide gel electrophoresis using the method of Swank and Munkrees (1971) was used to characterize the resulting peptides (Figure 3).

Peptides in the 3000–8000 range were detected during the hydrolysis of zein either in toluene or in ethyl acetate. A 6 times longer incubation period was necessary, however, to reach the same level of hydrolysis in the latter solvent. These results confirmed that PEG$_{2000}$–papain hydrolyzed zein to a significant extent in organic media, producing peptides with molecular masses comparable to those observed when zein was suspended in water (Virtanen et al., 1950) or solubilized in a homogeneous system containing 70% ethanol and 30% aqueous buffer (Saito et al., 1988).

Papain-catalyzed incorporation of lysine and phenylalanine into zein was carried out in toluene containing 1% added water (v/v), using the amino acid ester or amide as the nucleophile acceptor (Table III). With Lys-OMe, the lysine content of Lys–plastein increased from 0.15% to 3.5%, whereas a 3-fold higher enrichment was obtained with Lys-NH$_2$. With Phe-NH$_2$, the phenylalanine content of the final product was 4 times higher than in the zein hydrolysate. Comparable results were obtained in ethyl acetate containing 6% water (v/v). The amino acid

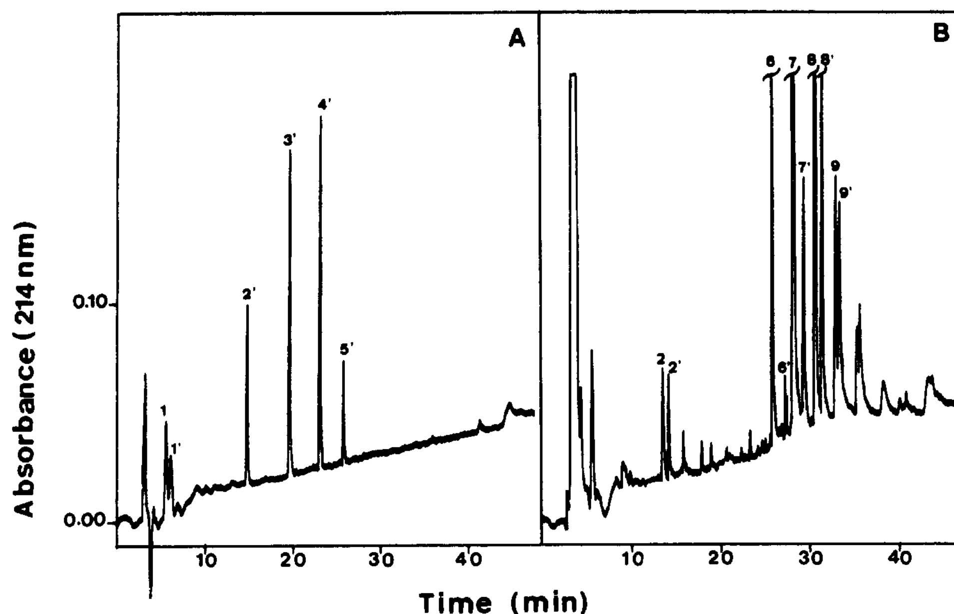

Figure 4. Reversed-phase HPLC analysis of methionine oligomer standards (A) and of the reaction mixture obtained after 20-h incubation of Met-NH_2 (10 mM) with PEG_{2000}–papain (17 μM), in benzene (B). The reaction was performed in the presence of 0.8% DIPEA (v/v), 0.2% mercaptoethanol (v/v), 0.4% methanol (v/v), and 1% added water (v/v). The chromatogram obtained showed a linear gradient from 5 to 90% acetonitrile in 0.05% trifluoroacetic acid (v/v) with a flow rate of 1 mL/min for 60 min. Formate was eluted in the first peak of each chromatogram. The following methionine derivatives were eluted: (1) Met-NH_2, (1′) Met-OH, (2) $(Met)_2$-NH_2, (2′) $(Met)_2$-OH, (3′) $(Met)_3$-OH, (4′) $(Met)_4$-OH, (5′) $(Met)_5$-OH, (6) $(Met)_6$-NH_2, (6′) $(Met)_6$-OH, (7) $(Met)_7$-NH_2, (7′) $(Met)_7$-OH, (8) $(Met)_8$-NH_2, (8′) $(Met)_8$-OH, (9) $(Met)_9$-NH_2, and (9′) $(Met)_9$-OH, as determined by logarithmic regression and mass spectrometry.

Table III. Amino Acid Composition (Weight Percent) of Lys- and Phe-Plasteins of Zein[a]

	zein	Lys-OMe plastein	Lys-NH2 plastein	Phe-NH2 plastein	control[b]
Asx	4.72	5.07	3.49	4.85	5.16
Met	0.39	0.87	0.90	0.78	0.25
Thr	2.70	2.58	2.08	2.24	2.35
Ser	5.73	6.76	4.64	4.64	6.12
Glx	20.69	18.00	17.05	15.66	19.78
Pro	11.70	9.32	9.80	8.99	12.15
Gly	1.73	2.72	2.99	2.47	2.50
Ala	13.39	11.87	10.68	10.56	12.82
Cys	0.57	0.41	nd[c]	nd	0.83
Val	3.75	4.02	4.67	3.77	4.23
Ile	3.71	3.66	3.94	3.27	3.87
Leu	19.37	17.46	17.52	15.04	18.65
Tyr	3.33	3.84	3.98	3.50	3.81
Phe	5.47	4.69	4.55	**22.07**	5.16
Lys	0.15	**3.53**	**10.37**	0.58	0.47
His	0.92	0.88	0.86	0.64	1.27
Arg	1.04	1.47	1.64	1.25	1.37
yields, %	100	93	80	100	85

[a] Zein (1 mg) dissolved in 0.5 mL of toluene containing 8% ethanol (v/v) was incubated at 37 °C for 20 h in the presence of PEG_{2000}–papain (17 μM), 0.8% DIPEA (v/v), 0.2% mercaptoethanol (v/v), 1% added water (v/v), and 10 mM amino acid derivative. In the case of Lys-OMe, ethanol was replaced by methanol. The final protein hydrolysate was purified by dialysis and the amino acid composition determined. [b] Enzymatic hydrolysate obtained under the same reaction conditions but without any amino acid derivative. [c] nd, not determined.

enrichment seems to be much more efficient in aqueous media (Aso et al., 1974). The fact that higher amino acid incorporation values were obtained in zein than in BSA may be due to the absence of any polymerization side reaction. It can be assumed that ethanol or methanol used at high concentrations for zein modification acts as a potent nucleophile acceptor and is probably involved in transesterification reactions which are in competition with peptide bond synthesis. Since cosolvents of the medium are able to interact with the enzyme, no conclusions can be drawn as to whether the protein substrate affected the incorporation yield.

Amino Acid Polymerization. To determine the effects of the organic solvent on the polymerization of the amino acid derivative, the experiments were repeated under the same conditions but without the protein substrate, because it was easier to characterize the final product by HPLC in the absence of peptides resulting from protein digestion. In the case of methionine amide, as shown in Figure 4, the results of the analysis of the resulting reaction solution were compared to the data obtained on a mixture of synthetic methionine oligomers (Gaertner and Puigserver, 1984). More than 90% of the initial substrate was polymerized, resulting mainly in oligomers having 5–10 residues, as determined by their retention times on HPLC and molecular masses. Peaks 7, 7′, 8, and 8′ were characterized by fast atom bombardment mass spectrometry and corresponded to Met_7-NH_2 (calculated M + H, m/z value 935.3; actual value 935), Met_7-OH (calculated M + H, m/z value 936.3; actual value 936), Met_8-NH_2 (calculated M + H, m/z value 1066.3; actual value 1066), and Met_8-OH (calculated M + H, m/z value 1067.3; actual value 1067), respectively. Comparable results involving the utilization of more than 90% of the initial substrate were obtained with Lys-NH_2 and to a lesser extent with Lys-OMe. On the other hand, only 44% and 30% of L-Met-OEt and Phe-NH_2, respectively, were used during the papain-catalyzed polymerization reaction. D-Met-NH_2 was definitely unsuitable for peptide bond synthesis. The high efficiency of lysine derivatives in papain-catalyzed polymerization in organic media strongly supports the idea that this reaction also occurred in the presence of a protein substrate, leading to some highly soluble polymerized material that may have been subsequently lost during the dialysis step.

The high hydration level of the enzyme under these conditions in the absence of the protein substrate confers high conformational mobility to the enzyme which is thus able to accommodate these substrates for a polymerization reaction. It is worth noting here that no such reaction

occurred at the low water concentration of 0.1% (v/v) (Gaertner et al., 1990).

Though papain exhibits a broad specificity with regard to the nature of the amino acid residues, it is worth mentioning that both Met and Lys derivatives are efficient substrates of papain in water/organic media, while the oligomerization reaction in water was observed especially with neutral and aromatic derivatives (Anderson and Luisi, 1979; Fruton, 1981). It can therefore be assumed that increasing the water concentration to 1% (v/v) yields either a multiphasic reaction medium or a suspension of microparticulates (Kahn and Halling, 1992).

One intriguing aspect of catalysis in low water environments is that PEG-modified proteases apparently do not show the same behavior as catalyst toward protein substrates. PEG–chymotrypsin did not catalyze any proteolysis in the presence of 1% added water, while the modified enzyme was efficient in peptide synthesis (Gaertner and Puigserver, 1988; Gaertner et al., 1991). PEG–trypsin was active (Gaertner and Puigserver, 1991), but the amino acid derivative (Phe-NH_2) was found to be essential for catalysis. Our results demonstrate that in the case of papain only the concentration of water in the reaction mixture is critical for the proteolysis and polymerization of hydrophobic, or basic (Ferjancic et al., 1991), as well as acidic (Uemura et al., 1990) amino acid derivatives. Moreover, papain seems to be a useful and efficient tool for incorporating amino acids into proteins suspended in organic media and thus opens a new pathway as a means of modifying food proteins for nutritional or functional purposes. In addition to the presence of water, which in organic media affects the ability of the enzyme to accommodate these substrates for hydrolysis or peptide bond formation, the presence of an extended active site in the biocatalyst is also an important parameter promoting the polymerization reaction (Ferjancic et al., 1991). The latter feature is now under investigation with a view to obtaining further information about the enhancement of the papain catalytic properties in organic solvents.

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