(Figure 6), suggesting that the contribution of tryptophan is greater than that of tyrosine (Teale, 1960).

CD Spectrum. The CD spectrum of the protein in the region 200–260 nm is given in Figure 7. It consisted of a shoulder at 208 nm and minimum at 224 nm. Calculation of the α helical content of the 2S protein from its ellipticity value at 208 nm using the equation of Greenfield and Fasman (1969) showed the value to be 35%. When the proportion of the β structure was estimated by the method of Sarkar and Doty (1966) taking a mean residue ellipticity value of 23 000° cm^2/dmol at 218 mm for 100% β structure, the proton was found to contain 38% β structure. Thus, the major structures appeared to be α helix and β structure. Many other low molecular weight vegetable proteins are known to consist predominantly of α helix and β structure (Schwenke et al., 1973; Madhusudhan, 1984).

LITERATURE CITED

Adler, A. J.; Greenfield, N. J.; Fasman, G. D. "Methods in Enzymology"; Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: New York, 1973; Vol. 27, p 675.
Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108.
Laemmeli, U. K. *Nature (London)* **1970**, *227*, 680.
Layne, E. "Methods in Enzymology"; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1957; Vol. 3, p 447.
Madhusudhan, K. T. Ph.D. Thesis, Mysore University, Mysore, 1984.
Montgomery, R. *Biochem. Biophys. Acta* **1961**, *48*, 591.
Reisfeld, R. A.; Lewis, I. J.; Williams, D. E. *Nature (London)* **1962**, *195*, 281.
Sarkar, P. K.; Doty, P. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *55*, 981.
Schachman, H. K. "Ultracentrifugation in Biochemistry"; Academic Press: New York, 1959.
Schwenke, K. D.; Raab, B.; Uhling, J.; Tkocz, H.; Behlke, J.; Bottger, M.; Freimuth, U. *Die Nahrung* **1973**, *17*, 791.
Shanmugasundaram, T.; Venkataraman, L. V. *J. Biosci.* **1982**, *4*, 419.
Taussky, H. H.; Shorr, E. *J. Biol. Chem.* **1953**, *202*, 675.
Teale, F. W. J. *Biochem. J.* **1960**, *76*, 381.
Youle, R. J.; Huang, A. H. C. *Am. J. Bot.* **1981**, *68*, 44.
"Wealth of India"; Council of Scientific and Industrial Research; New Delhi, 1952; Vol. 3.

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Hydrolysis of Poly-L-methionyl Proteins by Some Enzymes of the Digestive Tract

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Polymerization of L-, [^{35}S]-L-, and D,L-methionine on casein by the *N*-carboxy anhydride method allowed us to prepare protein derivatives in which 50–70% of the lysyl residues were acylated by methionine polymers of an average chain length of 5–12 residues. In vitro digestibility of resulting poly-methionylcaseins was investigated by successive incubation with pepsin, pancreatic endopeptidases, and intestinal aminopeptidase and subsequent amino acid analysis of hydrolysates. Methionine was readily released from covalently attached polymers although hydrolysis was significantly decreased with increasing chain length. Hydrolysis of casein itself was independent of the extent of modification of the lysyl residues whereas D,L-polymethionine chains were not well hydrolyzed. Analysis of peptic and chymotryptic hydrolysates using high-performance liquid chromatography showed that trimethionine and comparable amounts of di-, tri- and tetramethionine were released by each of the corresponding enzymes, respectively. These peptides were in turn good substrates for intestinal aminopeptidase. Polymethionyl proteins must therefore have potential nutritional applications.

INTRODUCTION

The methionine content of various food proteins has recently been shown to be enhanced by covalent attachment of poly-L-methionine through the *N*-carboxy anhydride method (Puigserver et al., 1982; Gaertner and Puigserver, 1984a). Protein α- and ϵ-amino groups are known to act as initiators in the polymerization of *N*-carboxy-α-amino acid anhydrides, leading to the formation of protein derivatives in which a number of poly(amino acid) chains of variable length are covalently attached to some of the lysyl residues (Glazer et al., 1962; Wellner et al., 1963). By contrast, the *N*-hydroxysuccinimide ester method resulted in the exclusive formation of a single isopeptide bond between each lysyl ϵ-amino group and the amino acid (Puigserver et al., 1979a).

The *N*-carboxy anhydride method has the obvious advantage of considerably increasing the amount of covalently linked amino acids even in proteins with low lysine levels. However, since as much methionine as 30% of casein weight could be covalently attached in the form of polymethionine of an average chain length of 8 residues, solubility of the protein derivative was drastically decreased (Gaertner and Puigserver, 1984a). Initial rates of hydrolysis by α-chymotrypsin and trypsin in vitro were decreased as compared to untreated casein, probably because of both steric hindrance preventing an efficient hydrolysis of the polypeptide chain and the presence of acylated lysyl residues inhibiting trypsin action. All these findings might suggest that covalent attachment of polymethionine chains to casein would result in a significant decrease of the overall digestibility of the protein derivatives in vivo. It was therefore important to test this hypothesis to see whether or not such a procedure was of value in improving protein quality.

The aim of this study was to determine in vitro digestibility of casein modified by using the *N*-carboxy anhydride method under different experimental conditions and subsequent availability of the polymerized methionine. To determine the hydrolytic processes for covalently linked polymethionine chains, digestions with a number of individual enzymes were carried out and the resulting data compared to those previously obtained with the synthetic

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model isopeptides N^{ϵ}-oligo-L-methionyl-L-lysine (Gaertner and Puigserver, 1984b).

EXPERIMENTAL SECTION

Materials. Orthophosphoric acid (supra pure grade), acetonitrile Lichrosolv, and casein were from Merck, Darmstadt, FRG. L-Methionine and D,L-methionine were from Sigma Chemical Co., St. Louis, MO, while [^{35}S]-L-methionine (1000 Ci/mmol) was from Amersham Corp., Les Ulis, France. *N*-Hydroxysuccinimide ester of α-[(*tert*-butyloxy)carbonyl]-L-methionine as well as the L forms of Met_2 and Met_3 were supplied by Bachem Fine Chemicals, Bubendorf, Switzerland. Synthesis of Met_4 and Met_5 was carried out by the solid-phase procedure (Barany and Merrifield, 1980) as detailed by Gaertner and Puigserver (1984b). Chymotrypsin, trypsin, and pepsin were from Worthington Biochemical Corp., Freehold, NJ, whereas elastase was from Boehringer, Mannheim, FRG. The latter enzyme contained about 20% (w/w) contaminating carboxypeptidase A as estimated by activity determination using the synthetic ester substrate hippuryl-L-phenyllactate. Pure hog aminopeptidase N and calf pancreatic juice were gifts from S. Maroux, Marseille, and P. Thivend, Theix, France, respectively. Analytical grade chemicals and reagents were used throughout.

Preparation of Modified Proteins. *N*-carboxy-L-methionine anhydride was synthesized by reacting the amino acid, suspended in anhydrous tetrahydrofuran, with phosgene (Hirschmann et al., 1971) in a hood with good draft as previously described (Gaertner and Puigserver, 1984a). The samples of poly-L-methionylcasein were prepared by incubating a 5% solution of protein in a 0.1 M sodium citrate buffer (pH 6.5) with various amounts of the *N*-carboxy anhydride dissolved in tetrahydrofuran (Gaertner and Puigserver, 1984a). The resulting reaction mixture was vigorously stirred for 15 min at 4 °C while the pH was kept constant by manual addition of 0.1 N NaOH every 2–3 min. On the other hand, five successive additions of reagent to the 5% casein solution buffered with 0.1 M bicarbonate at pH 10.2 were made in order to modify all the protein lysyl residues. Each addition contained a 1.5:1.0 molar ratio of *N*-carboxy anhydride to amino groups (Gaertner and Puigserver, 1984a). Complete modification of the lysyl residues was also achieved with the *N*-hydroxysuccinimide ester of the (*tert*-butyloxy)carbonyl amino acid (Puigserver et al., 1979a). In the latter case, a single methionyl residue was covalently attached to each lysyl residue whereas under stepwise addition of the *N*-carboxy anhydride, polymerization of L-methionine on some of the casein amino groups cannot be precluded.

Poly-[^{35}S]-L-methionylcasein was prepared by reacting 500 mg of protein dissolved in 10 mL of a 0.1 M citrate buffer (pH 6.5) with radioactive *N*-carboxy-L-methionine anhydride synthesized from a mixture of both unlabeled and labeled amino acid (230 mg- and 0.3 μg-2 mCi, respectively).

All modified proteins were dialyzed extensively against water, freeze-dried, and finally used for in vitro digestibility studies without any further purification.

Amino Acid Analysis and Amino Group Determination. Control and modified caseins were hydrolyzed with distilled 5.6 N HCl at 100 °C for 24 h, and their amino acid composition was determined with a Beckman Model 120 C autoanalyzer equipped with an ICAP 10 computing system. By contrast, free amino acids released by enzymatic digestion of proteins in vitro were directly estimated with the autoanalyzer without prior acid hydrolysis. In the first case, however, methionine was evaluated in the form of methionine sulfone after performic acid oxidation of the protein (Hirs, 1956), while tryptophan was not determined.

The number of unreacted ϵ-amino groups of the lysyl residues was identified as homoarginine after a prolonged incubation of the protein with *O*-methylisourea at pH 10.5 (Kimmel, 1967). The number of guanidinated lysyl residues was determined with the amino acid analyzer as already described (Gaertner and Puigserver, 1984a).

In Vitro Enzyme Digestion Studies. Reagent control casein and modified casein samples were subjected to a successive digestion by hog pepsin, calf pancreatic juice, and hog intestinal aminopeptidase according to the method of Matoba et al. (1980) with slight modifications. Pancreatic juice, about 1.5 mg of protein/mL of a 0.05 M sodium phosphate buffer at pH 8.0, was activated by trypsin (1:10; w/w) at 0 °C for 3 h. The resulting tryptic, chymotryptic, elastolytic, and carboxypeptidasic A and B activities, as well as those of pepsin and aminopeptidase, were determined by using their specific synthetic substrates as detailed in a previous publication (Puigserver et al., 1979b). In a typical experiment, extensive enzymatic hydrolysis of casein and casein derivatives (about 1 μmol) included at first an incubation with pepsin (1:50; w/w) in 5 mL of 0.05 N HCl at 38 °C for 10 h. After neutralization with 1 N NaOH and addition of 1 mL of the activated pancreatic juice (1:20; w/w) buffered at pH 8.0, the reaction mixture was incubated at 38 °C and pH 8.0 for an additional 15-h period. Then, the reaction was stopped by boiling for 5 min and subsequent adjustment to pH 7. Digestion of casein samples was therefore completed by intestinal aminopeptidase N (1:500; w/w) at 37 °C for 20 h. After the hydrolysate was diluted 2-fold with a 0.2 M citrate buffer (pH 3.2), a 1-mL aliquot was assayed for amino acid. The use of high enzyme concentrations in such experiments resulted in some enzyme autolysis, leading to a significant release of almost all the amino acids. This was therefore taken into account to estimate casein digestion. Incubation of protein samples without enzyme showed that some nonenzymatic hydrolysis occurred since some new amino groups could be detected with 2,4,6-trinitrobenzenesulfonic acid (Fields, 1972). However, neither free amino acids nor short peptides were released, as estimated by automatic amino acid analysis.

Hydrolysis of casein derivatives (0.2 μmol) was also carried out separately with pepsin in 0.05 M HCl and individual pancreatic peptidases in a 0.1 M NH_4HCO_3 (pH 8.2) in order to precisely study digestion of poly-L-methionine covalently attached to the protein. After the reaction mixture was incubated with each enzyme (1:50; w/w) at 38 °C for 20 h, the reaction was stopped either by boiling for 5 min in the case of pepsin or by addition of glacial acetic acid (pH 3.0) for the remaining enzymes. The hydrolysate was centrifuged at low speed to remove any precipitated material, and the resulting supernatant (1 mL) was then filtered through a Sephadex G 15 column (1 × 60 cm). Peptides eluted with 0.1 M acetic acid were monitored at 206 nm with an LKB 2138 Uvicord S. Casein, Met_5, Met_3, Met_2, and methionine were used as references of known molecular weights. As indicated above, control experiments for nonenzymatic hydrolysis were also performed.

HPLC Separation of Peptides. Peptide fractions eluted from the Sephadex column were freeze-dried and further purified by HPLC. The Waters Associates system that was used consisted of an M 6000 A and an M 45 solvent-delivery unit, a M 720 solvent programmer, a U 6K injector, an M 441 ultraviolet spectrophotometer, a M 730 data module, and a Merck Lichrosorb C 18 reversed-

Table I. Extent of Modification of Casein with N-Carboxymethionine Anhydride

protein	sample	methionine content[a] g/100 g of casein	methionine content[a] mol/mol of casein	modified lysyl residues[b] %	modified lysyl residues[b] mol/mol of casein
L-methionylcasein	1[c]	9	16	95	11.4
poly-L-methionyl-casein	2	14	29	95	11.4
	3	19	41	56	6.7
	4	27	64	59	7.1
	5	29	69	62	7.4
	6	34	90	66	7.9
	7	39	110	72	8.6
poly-D,L-methionyl-casein	8	17	35	50	6.0

[a] Including the intrinsic methionyl content (4 residues/mol of whole control casein; M_r 23 kDa). [b] Determined as those residues that were not transformed into homoarginine by reaction of casein with O-methylisourea. Extent of modification was based on an average of 12 lysyl residues/mol of protein. [c] Sample 1 was modified by the N-hydroxysuccinimide ester method (Puigserver et al., 1979a) using a molar ratio of reagent to protein amino groups of 4:1. Sample 2 was modified by four successive additions of N-carboxy-L-methionine anhydride at pH 10.2 (1.5:1.0 molar ratio, each time) while samples 3, 4, and 6–8 were modified by a single addition of reagent at pH 6.5. Molar ratios of reagent were 4, 6, 8, 10, and 6 for samples 3, 4, and 6–8, respectively. Sample 5 was modified by a single addition of [^{35}S]-N-carboxy-L-methionine anhydride at pH 6.5 (7:1 molar ratio).

phase column (7 μm; 4 × 250 mm). Peptides were separated by a nonlinear gradient from 0 to 60% acetonitrile in 0.1% orthophosphoric acid (pH 2.2) at a constant flow rate of 1 mL/min and at room temperature. Detection was performed at 214 nm with a full scale A = 0.2–0.5. Orthophosphoric acid solution (pH 2.2) was first filtered through a 0.45-μm Millipore membrane before sonication for 15 min for degassing whereas acetonitrile was directly sonicated. Radioactive peptides separated from poly-[^{35}S]-L-methionylcasein digestion mixtures were collected in 1-mL fractions, and their radioactivity was then determined in a Beckman liquid scintillation spectrometer, Model 3800.

RESULTS

Characterization of Modified Caseins. The amount of methionine covalently attached to casein as well as the extent of modification of the lysyl residues was determined by amino acid analysis and ε-amino group determination (Table I). Complete acylation of protein ε-amino groups was readily achieved by either the N-hydroxysuccinimide ester method (Puigserver et al., 1979a) or successive additions of N-carboxy-L-methionine anhydride at alkaline pH (Puigserver et al., 1982; Gaertner and Puigserver, 1984a). Under polymerization conditions of the latter amino acid derivative at pH 6.5, only 50–70% of the lysyl residues was modified depending on the molar excess of reagent over the number of amino groups. In the most extensively modified protein derivative (sample 7), the average chain length of covalently attached poly-L-methionine was estimated to be in the range of 10–12 residues. Covalent attachment of methionine polymers has already been shown to significantly decrease the initial rates of hydrolysis of modified caseins by chymotrypsin or trypsin. It was therefore interesting to investigate the combined action of digestive enzymes of the upper alimentary tract.

Extensive Enzymatic Hydrolysis of Casein Derivatives. The digestibility of modified caseins was investigated in vitro by successive incubation with pepsin, activated pancreatic juice, and intestinal aminopeptidase. Amino acid analysis (Table II) of the resulting hydrolysates showed a good release of most amino acids, ranging from 40% for neutral amino acids to 80% for basic and aromatic residues, comparable to that of unmodified casein. By contrast, the poor release of acidic amino acids (<10%) should be related to the lack of acidic aminopeptidase in the incubation medium. Especially of interest was the 5-fold to 10-fold increase in free methionine resulting from poly-L-methionylcasein samples, thus indicating a rather good hydrolysis of the covalently attached polymers. However, the relative release of methionine (%) was found to be somewhat lower in highly modified caseins. A significant hydrolysis of poly-D,L-methionine polymers was also observed. The elution of Asn and Gln together with Ser and Thr under the chromatographic conditions used for automatic amino acid analysis (sodium citrate buffers) could probably explain the unexpected high levels of the latter two amino acids as compared to the others.

When all the lysyl residues of casein were modified by either the N-hydroxysuccinimide ester method (sample 1) or the N-carboxy anhydride method under stepwise addition conditions (sample 2), they were not released from the protein to the same extent. With the former casein derivative, in which each lysyl residue was acylated by a single methionine, about 50% of the lysyl residues (5.9 over 12) was released instead of about 20% in the latter derivative, which contained an average of two methionyl

Table II. Amino Acids Released from Control Casein and Casein Derivatives (mol/mol of Protein) by Pepsin, Activated Pancreatic Juice, and Pig Aminopeptidase N

amino acid	control casein	1	2	3	4	6	7	8	compn of control casein
Asp	1, 5	1, 1	1, 0	1, 1	<1	<1	<1	<1	12
Thr + Ser	11, 5	10, 5	9, 5	11	9, 7	9, 1	9, 2	8, 2	19
Glu	1, 3	1, 3	1, 0	1, 5	2, 2	1, 8	1, 5	1, 5	34
Pro	1, 5	1, 2	1, 7	<1	<1	<1	<1	<1	20
Gly	1, 8	1, 7	1, 2	1, 2	1, 4	1, 3	1, 3	1, 2	6
Ala	4, 1	3, 8	2, 8	2, 9	2, 3	3, 5	2, 4	3, 1	8
Val	3, 6	4, 3	3, 6	3, 6	4, 3	4, 9	4, 8	3, 9	14
Met	2, 4	*12, 4*	*16, 1*	*25, 4*	*28, 1*	*37, 7*	*37, 8*	*7, 5*	4
Ile	3, 3	3, 3	2, 9	2, 9	2, 4	2, 5	2, 6	2, 2	8
Leu	9, 9	10, 2	9, 8	9, 5	8, 9	8, 5	8, 5	7, 4	16
Tyr	5, 3	5, 6	6, 0	5, 4	1, 0	1, 9	1, 9	2, 1	7
Phe	5	5, 7	5, 9	5, 2	4, 1	5, 0	4, 9	3, 8	6
Lys	7, 7	5, 9	2, 7	5, 9	6, 9	5, 2	4, 7	4, 0	12
His	2, 5	2, 4	1, 9	2, 1	1, 0	1, 1	1, 0	1, 0	4
Arg	3, 7	3, 4	3, 7	3, 5	2, 5	3, 9	3, 8	3, 0	5
Met (%)[a]		90	55	62	44	41	32	16	

[a] [(Total methionine released − intrinsic methionine released)/total methionine covalently attached to casein] × 100.

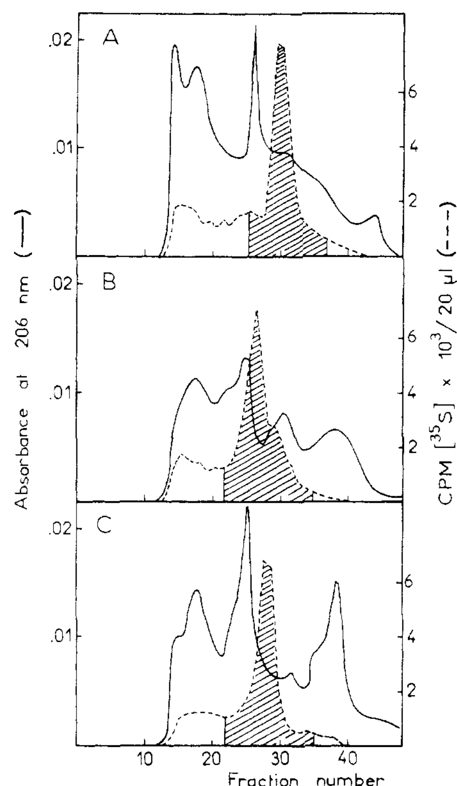

Figure 1. Filtration through a Sephadex G 15 column (1 × 60 cm) of peptic (A), chymotryptic (B), and elastolytic (C) hydrolysates of poly[^{35}S]-L-methionylcasein. Shaded areas correspond to the low molecular weight peptides that were pooled for HPLC analysis.

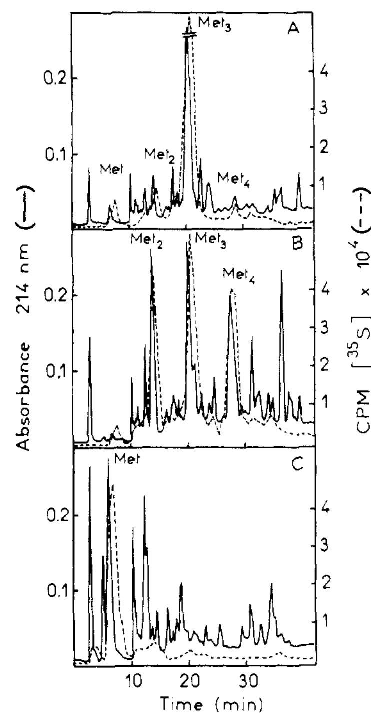

Figure 2. HPLC elution profiles of peptic (A), chymotryptic (B), and elastolytic (C) digests of poly[^{35}S]-L-methionylcasein. Methionine and related oligomers were eluted from the 7-µm Lichrosorb RP 18 column (4 mm × 25 cm) by a 35-min nonlinear gradient from 0.1% orthophosphoric acid (aqueous solution) to acetonitrile 0.1% orthophosphoric acid (60–40%) with a flow rate of 1 mL/min. Aliquots (0.2 mL) removed from the collected fractions (1 mL) were assayed for radioactivity.

residues per lysyl ε-amino group. In caseins modified under polymerization conditions (samples 3, 4, 6, and 7), lysine availability decreased when the polymethionine chain length increased.

Since methionyl–methionine bonds were hydrolyzed under extensive digestion conditions, it was decided to investigate more precisely the hydrolysis of covalently attached polymethionine chains.

Hydrolysis of Methionine Polymers. Pepsin, trypsin, chymotrypsin, elastase, and aminopeptidase were separately incubated with casein derivatives, and the resulting hydrolysates were analyzed for free methionine and methionine peptides in order to examine the hydrolysis of polymethionine chains covalently attached to the lysyl residues of casein. Large peptides and nonhydrolyzed proteins were separated from short peptides by filtration of the incubation mixtures through a Sephadex G 15 column prior to resolution of methionine and related oligomers up to Met_5 by means of an HPLC system as detailed by Gaertner and Puigserver (1984b). Nonenzymatic hydrolysis of protein samples was not observed since neither free amino acids nor free peptides could be detected with an autoanalyzer.

Figure 1 shows the elution profile of peptic, chymotryptic, and elastolytic digestion mixtures of poly-[^{35}S]-L-methionylcasein (Table I, sample 5). Fractions containing short radioactive peptides (shaded peaks) released by hydrolysis of the covalently bound polymethionine chains were pooled and chromatographed on a reversed-phase HPLC column as illustrated in Figure 2. Each isolated radioactive peptide could therefore be identified by comparing its retention time to that of pure synthetic model peptides (Gaertner and Puigserver, 1984b). Identification was also confirmed by amino acid analysis of the collected material. Methionine was determined as methionine sulfone after performic acid oxidation and subsequent acid hydrolysis of the fractionated peptides.

Polymethionine chains covalently attached to the lysyl residues of casein were essentially hydrolyzed by pepsin to Met_3 and by chymotrypsin to Met_2, Met_3, and Met_4. The important release of free methionine observed with elastase was probably due to the contaminating carboxypeptidase A activity. On account of its narrow specificity, trypsin was found not to hydrolyze methionine polymers while aminopeptidase N was only slightly active on the whole protein. The overall results derived from enzymatic digestion of covalently linked methionine polymers were in good agreement with those obtained with the model isopeptides N^{ϵ}-oligo-L-methionyl-L-lysine (Gaertner and Puigserver, 1984b).

The peptide material eluted under each peak in Figure 2 was collected and the radioactivity estimated as indicated in the Experimental Section. Although collecting fractions from an HPLC column is known to stretch the peaks (Kessler, 1983), leading to possible contamination by radioactive materials with slightly different retention times, a very good correlation was found between peptide amounts derived from radioactivity determinations and peak areas. It was therefore possible to quantitatively estimate the peptides released by enzymatic digestion of methionine polymers covalently attached to casein as influenced by the extent of modification of the protein.

Quantitative Estimation of Released Peptides. This was readily achieved by means of the HPLC system since a direct relationship has already been shown to exist between computer-integrated peak areas and the amount of methionine and related oligomers in the 2–40-nmol range (Gaertner and Puigserver, 1984b). Figure 3 shows the time course of pepsin and chymotrypsin hydrolysis of polymethionine chains covalently linked to casein as a result

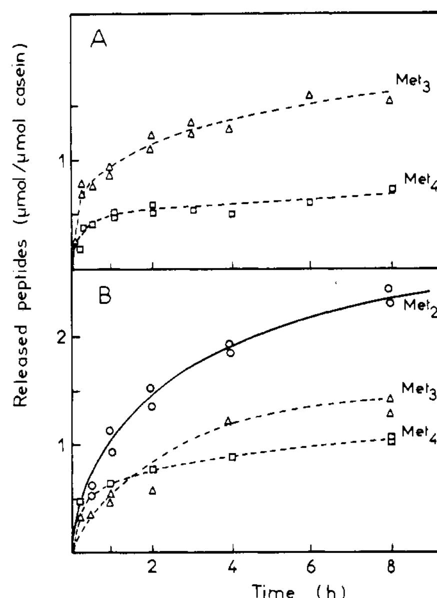

Figure 3. Time course hydrolysis of polymethionine covalently attached to casein (sample 3) by pepsin (A) and chymotrypsin (B). Casein derivative (0.2 μmol) was hydrolyzed at 38 °C by each enzyme (1:50, w/w) for increasing periods of time. After the reaction was stopped, soluble material was filtered through the Sephadex G 15 column (1 × 60 cm) and the resulting low molecular weight fractions were then analyzed by means of the HPLC system.

Table III. Methionine Release[a] in the Form of Free Amino Acid and Related Peptides by Pepsin, Chymotrypsin, and Elastase

enzyme	released methionine, %						
	1	2	3	4	6	7	8
pepsin			40	26	19	19	4
chymotrypsin		7	40	44	34	27	7
elastase	10	13	36	40	29	28	15

[a] Samples are those indicated in Table I. Digestion was performed at 38 °C for 20 h with 1:50 (w/w) enzyme.

of the polymerization of 4 mol of *N*-carboxy-L-methionine anhydride per amino group at pH 6.5 (sample 3, Table I). As indicated, the rate of Met_3 released by pepsin was higher than that of Met_4, while the casein derivative was essentially hydrolyzed by chymotrypsin to Met_2 and to a lesser extent to both Met_3 and Met_4. After a 8-h period of enzyme digestion, about 30% and 40% of covalently linked methionine were released from polymethionine chains by pepsin and chymotrypsin, respectively.

By this type of kinetic approach, extents of hydrolysis of a number of differently modified caseins were estimated after 20 h of incubation at 37 °C with pepsin, chymotrypsin, and elastase (1:50; w/w). As shown in Table III, the relative release of methionine (%) in the form of free amino acid or related oligomers was influenced by the average chain length of attached polymers. A 2-fold decrease in digestibility by pepsin was observed between casein derivatives 3 and 7 in which 37 and 106 methionyl residues were covalently linked, leading to polymers of an average chain length of 5–6 and 12–13 residues, respectively. Although slightly smaller, a comparable decrease was also observed with chymotrypsin and elastase.

However, when less than 2–3 methionyl residues were attached per lysine ε-amino group, as in sample 2, almost no methionine was released. The fact that elastase hydrolyzed some methionyl–methionine bonds in sample 1, in which all the amino groups were acylated by a single methionine, is certainly due to the cleavage of the polypeptide chain in the N-terminal region and subsequent hydrolysis by contaminating carboxypeptidase A. Although poly-D,L-methionine chains were weakly hydrolyzed by pepsin, chymotrypsin, and elastase, covalent attachment of racemic methionine to proteins did not seem to be a good way for improving the nutritional value of food proteins.

Analysis of pepsin digestion mixtures from the casein derivatives 3, 4, 6, and 7 (Table III) indicated that, whatever the average chain length of covalently linked polymethionine (5–6 to 12–13 residues), 60–70% of total methionine was released as Met_3, 10–20% as Met_4, and less than 10% as Met_2. With chymotrypsin, the following values were obtained: 30–40% of Met_3 as well as of Met_2 and 20–30% as Met_4. In both cases, Met_4 was the largest peptide identified in the hydrolysates.

DISCUSSION

Our results from in vitro enzyme digestion studies of casein derivatives clearly indicated that polymerization of L-methionine on casein by the *N*-carboxy anhydride method did not result in any important decrease in hydrolysis of the polypeptide chain by digestive enzymes. Moreover, the polymethionine of an average chain length of 12 residues, which was covalently linked to the lysyl residues, was also readily hydrolyzed although the release of methionine was inversely correlated with the chain length. Nonenzymatic hydrolysis of covalently attached polymethionine chains was actually found not to be significant under the experimental conditions used throughout this study. It is nevertheless worth stressing here that our in vitro digestion method, which is based on the estimation of amino acids released from modified proteins, did not take into account the hydrolysis of peptide bonds that did not give rise to free amino acids or short peptides.

The relatively high hydrolysis of poly-D,L-methionine polymers is noteworthy and strongly suggests the existence of a number of sequences made of at least 3 or 4 adjacent L-methionyl residues, as a result of the random polymerization of the *N*-carboxy-α-amino acid anhydride, rather than an alternate sequence of D and L residues. Since the ratio of D- over L-methionine in covalently attached polymers has never been precisely estimated, a preferential polymerization of the L isomer vs. D isomer cannot be ruled out. Hydrolysis of poly-D,L-methionine polymers may also be due to some intrinsic activity of digestive enzymes toward D peptide bonds. Although this type of enzyme-catalyzed hydrolysis is still poorly documented (Tsuyuki et al., 1956; Katchalski et al., 1964; Simons and Blout, 1964), Lundblad et al. (1975) have more recently provided some evidence for a weak hydrolysis of poly-D-lysine by pancreatin (Sigma Grade III), as assayed by pH-stat titration. On the other hand, both intestinal absorption and enzymatic hydrolysis of a D-amino acid containing dipeptide has been found to be impaired as compared to the L-dipeptide (Burston et al., 1972).

The already reported efficient hydrolysis of the isopeptide bond between the first attached methionyl residue and the ε-amino group of lysyl residues by intestinal membrane-bound aminopeptidase N (Puigserver et al., 1979b) was confirmed in this study (see Table II, sample 1). However, in spite of the broad specificity and high hydrolytic activity of this enzyme, one of the major peptidases of the intestinal mucosa (Kenny and Booth, 1978; Feracci et al., 1981), it was found to be slightly effective in releasing methionine from casein derivatives prior to digestion by endopeptidases. A poor accessibility of aminopeptidase to the scissile bonds rather than unfilled specificity requirements may explain this finding.

Another point of interest concerns digestion of covalently attached polymethionine chains by pepsin and chymotrypsin. All the peptides resulting from their hydrolysis, Met_3 in the former case and a mixture of Met_2, Met_3, and Met_4 in the latter, were expected from their well-known specificity. Pepsin exhibits a rather large specificity for peptide bonds C-terminal to aromatic residues, leucine, methionine, and glutamic acid (Tang, 1963), whereas chymotrypsin hydrolytic activity is essentially directed toward aromatic residues and to a much lesser extent to leucine and methionine (Neil et al., 1966). Furthermore, results from the present study are quite consistent with those obtained with model peptides and isopeptides (Gaertner and Puigserver, 1984b). Thus, pepsin was found to essentially release tripeptidyl units from the N-terminus of covalently linked methionine polymers whatever their chain length. Chymotrypsin preferentially hydrolyzed the methionyl–methionine bond preceding the isopeptide bond releasing di-, tri-, and tetramethionine. It is noteworthy that no peptide larger than tetramethionine was identified in polymethionylcasein hydrolysates. This could be due either to a stepwise formation of smaller peptides as already observed with other endopeptidases (Sass and Thiemann, 1973) or to the fact that medium-sized released oligopeptides were quite rapidly broken down on account of the high enzyme concentration in the incubation mixture (Katchalski et al., 1964). All the released peptides could be directly absorbed through the intestinal barrier in vivo or subsequently hydrolyzed by the exopeptidases. Methionine or related short oligomers, which were still linked to the lysyl residues, would be released by aminopeptidase. However, as long as a lysyl residue is not deacylated, its absorption is expected to be significantly delayed.

Since elastase is known to hydrolyze peptide bonds involving neutral amino acids (Hartley and Shotton, 1971), its activity toward covalently attached methionine polymers was not therefore surprising. However, no hydrolysis of the model isopeptides N^{ϵ}-oligo-L-methionyl-L-lysine has previously been observed (Gaertner and Puigserver, 1984b). This discrepancy may result from the fact that covalently linked methionine oligomers were short enough to lead to some disturbing electrostatic interactions with the hydrolytic enzyme. A possible more efficient hydrolysis of the polymethionine chain attached to the protein N-terminal amino acid rather than to the lysyl residues cannot be ruled out since methionine was also released from slightly modified caseins.

Successive hydrolysis of proteins by gastric pepsin, activated pancreatic juice, and intestinal aminopeptidase was considered to be a good means for investigating protein digestibility. However, exclusive estimation of the released amino acids to evaluate the extent of protein digestion is known to have a number of drawbacks. Among these is the fact that the first steps of protein digestion cannot be taken into account and that the inhibition of enzyme activity by reaction products as well as the absence of the complete diffusion of hydrolysis products in experiments involving dialysis cannot be prevented (Robbins, 1978; Vachon et al., 1982). The use of a broad range of hydrolases is actually known to result in a rather good simulation of the in vivo enzymatic digestive process. However, numerous studies performed with a restricted number of enzymes have already attempted to correlate in vitro and in vivo digestibility of proteins (Akeson and Stahmann, 1964; Hsu et al., 1977). More recently, an in vitro system using immobilized enzymes was found to have the additional advantage of preventing enzyme autolysis, therefore leading to more accurate results (Porter et al., 1984). However, this procedure is certainly not the most convenient for insoluble proteins as polymethionylcaseins. Whatever the method used, in vitro digestion studies are generally considered to lead to a good preliminary evaluation of both protein quality and amino acid availability and to detect any change in digestibility resulting from protein modification (Stahmann and Woldegiorgis, 1975).

The quite good hydrolysis of polymethionine chains covalently attached to casein under our experimental conditions led us to consider such protein derivatives as new products with potentially interesting nutritional properties. For instance, whereas poly-L-methionine was found to have almost no nutritional value because of its insolubility in aqueous phase (Boebel and Baker, 1982), it became an efficient source of methionine when covalently bound to casein (manuscript in preparation). Since poly-[^{35}S]-L-methionine was also reported not to be digestible at all in sheep (Downes et al., 1970), it may be expected that covalent attachment of polymethionine chains to proteins will considerably improve methionine availability. These protein derivatives will also be quite interesting to study regarding their resistance to rumen proteolysis.

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Registry No. L-Methionine, 63-68-3; D,L-methionine, 59-51-8; lysine, 56-87-1; pepsin, 9001-75-6; endopeptidase, 9001-92-7; aminopeptidase, 9031-94-1.

LITERATURE CITED

Akeson, W. R.; Stahmann, M. A. *J. Nutr.* **1964**, *83*, 257–261.
Barany, G.; Merrifield, R. B. "The Peptides: Analysis, Synthesis and Biology"; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1980; Vol. II, pp 1–284.
Boebel, K. P.; Baker, D. H. *J. Nutr.* **1982**, *112*, 1130–1132.
Burston, D.; Addison, J. M.; Matthews, D. M. *Clin. Sci.* **1972**, *43*, 823–837.
Downes, A. M.; Reis, P. J.; Sharry, L. F.; Tunks, D. A. *Br. J. Nutr.* **1970**, *24*, 1083–1089.
Feracci, H.; Benajiba, A.; Gorvel, J. P.; Doumeng, C.; Maroux, S. *Biochim. Biophys. Acta* **1981**, *658*, 148–157.
Fields, R. *Methods Enzymol.* **1972**, *25B*, 464–468.
Gaertner, H. F.; Puigserver, A. J. *J. Agric. Food Chem.* **1984a**, *32*, 1371–1376.
Gaertner, H. F.; Puigserver, A. J. *Eur. J. Biochem.* **1984b**, *145*, 257–263.
Glazer, A. N.; Bar-Eli, A.; Katchalski, E. *J. Biol. Chem.* **1962**, *237*, 1832–1838.
Hartley, B. S.; Shotton, D. M. "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. III, pp 332–337.
Hirschmann, R.; Schwann, H.; Strachan, R. G.; Schoenewaldt, E. F.; Barkemeyer, H.; Miller, S. M.; Conn, J. B.; Garsky, V.; Veber, D. F.; Denkewalter, R. G. *J. Am. Chem. Soc.* **1971**, *93*, 2746–2754.
Hirs, C. H. W. *J. Biol. Chem.* **1956**, *219*, 611–621.
Hsu, H. W.; Vavak, D. L.; Satterlee, L. D.; Miller, G. A. *J. Food Sci.* **1977**, 42, 1269–1273.
Katchalski, E.; Sela, M.; Silman, H. I.; Berger, A. "The Proteins", 2nd ed.; Neurath, H., Ed.; Academic Press: New York, 1964; Vol. II, pp 519–526.
Kenny, A. J.; Booth, A. G. *Assays Biochem.* **1978**, *14*, 1–44.
Kessler, M. J. *J. Chromatogr.* **1983**, *255*, 209–217.
Kimmel, J. R. *Methods Enzymol.* **1967**, *11*, 584–589.
Lundblad, G.; Elander, M.; Manhem, L. *Acta Chem. Scand.* **1975**, *B29*, 937–947.
Matoba, T.; Doi, E.; Yonezawa, D. *Agric. Biol. Chem.* **1980**, *44*, 2323–2328.

Neil, G. L.; Niemann, C.; Hein, G. E. *Nature* **1966**, *210*, 903–907.
Porter, D. H.; Swaisgood, H. E.; Catignani, G. L. *J. Agric. Food Chem.* **1984**, *32*, 334–339.
Puigserver, A. J.; Sen, L. C.; Gonzalez-Flores, E.; Fenney, R. E.; Whitaker, J. R. *J. Agric. Food Chem.* **1979a**, *27*, 1098–1104.
Puigserver, A. J.; Sen, L. C.; Clifford, A. J.; Feeney, R. E.; Whitaker, J. R. *J. Agric. Food Chem.* **1979b**, *27*, 1286–1293.
Puigserver, A. J.; Gaertner, H. F.; Sen, L. C.; Feeney, R. E.; Whitaker, J. R. *Adv. Chem. Ser.* **1982**, *No. 198*, 149–167.
Robbins, R. C. *Int. J. Vit. Nutr. Res.* **1978**, *48*, 44–53.
Sass, R.; Thiemann, W. *Biopolymers* **1973**, *12*, 535–539.
Simons, E. R.; Blout, E. R. *Biochim. Biophys. Acta* **1964**, *92*, 197–199.
Shahmann, M. A.; Woldegiorgis, G. "Nutritional Quality of Foods and Feeds"; Friedman, M., Ed.; Marcel Dekker: New York, 1975 Part I, pp 211–234.
Tang, J. *Nature* **1963**, *199*, 1094–1095.
Tsuyuki, E.; Tsuyuki, H.; Stahmann, M. A. *J. Biol. Chem.* **1956**, *222*, 479–485.
Vachon, C.; Gauthier, S.; Jones, J. D.; Savoie, L. *Nutr. Res.* **1982**, *2*, 675–688.
Wellner, D.; Silman, H. J.; Sela, M. *J. Biol. Chem.* **1963**, *238*, 1324–1331.

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Selective Thermal Denaturation as a Method of Preparative Isolation of 11S Globulins from Plant Seeds

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A method has been developed for isolation of 11S globulins from soybeans, broad beans, peas, sunflower seeds, and oats based on the principle of selective thermal denaturation. The preparation isolated from defatted soy meal contains 95–97% of 11S globulin according to sedimentation velocity measurements, while its yield amounts to 1–2 g/100 g of defatted meal. The preparation isolated from defatted meal of sunflower seeds contains 95–97% of 11S globulin, and its yield is 3–5 g/100 g of meal. The preparations of 11S globulin from broad beans, peas, and oats were isolated without preliminary defatting of the meal. The preparation of 11S globulin from oat seeds is of the same purity as those isolated from soybeans and sunflower seeds, while its yield accounts for 0.2–0.5 g/100 g of meal. The preparations of 11S globulins isolated from broad beans and peas contain no impurities, according to the sedimentation data. The yield of preparations is equal to 0.5 g/100 g of meal.

INTRODUCTION

The fractionation of proteins using the principle of selective thermal denaturation is well-known. Specifically, this principle is used for isolating enzymes (Kochetkov, 1980). It was also employed for isolating 11S globulin from soybeans (Osborne and Campbell, 1898), 13S globulin from sesame seeds (Hasegama et al., 1978), and 11S globulin from broad beans (Schlisier and Manteuffel, 1981). In the latter case it was shown that the isolated preparation is identical immunoelectrophoretically and serologically with the native 11S globulin.

EXPERIMENTAL SECTION

11S globulin from soybeans was isolated from defatted soy meal Soya Fluff 200 W (Central Soya International). 11S globulin from sunflower seeds was isolated from defatted meal (cultivar Yubileinyi-60). 11S globulins from broad beans (cultivar Orlovski-41), peas (cultivar Orlovski-3), and oats (unknown cultivar) were isolated from nondefatted meal.

Standard preparations of 2.8S, 7S, and 11S globulins were isolated from defatted soy meal according to the methods developed by Vaintraub and Shutov (1969) and Thanh and Shibasaki (1976). The preparation of 2.8S globulin was homogeneous according to velocity sedimentation, chromatography, and gel electrophoresis. The preparation of 7S globulin was sedimentationally homogeneous. The preparation of 11S globulin was purified by the chromatographic method on hydroxyl apatite (Wolf and Sly, 1965). According to sedimentation velocity measurements, it contained ~90% of 11S globulin, with an impurity having a sedimentation coefficient of 17S. The globulin fraction of soybeans was isolated by the isoelectric precipitation method (Wolf, 1972). According to sedimentation velocity measurements, this preparation contained 2S, 7S, 11S, and 15S components in a weight ratio 1:7.5:5.7:1.2. Chicken lysozyme (Type A, Biokhimreactiv; Olaine, USSR) with an activity of 26 000 units was used as calorimetric standard without additional purification.

Auxiliary materials (buffer salts, sodium chloride, guanidine hydrochloride (GuHCl), 2-mercaptoethanol, sodium azide) were of analytical grade. All the solutions were prepared with distilled water.

Preparation of Solutions. Stock aqueous solutions of 10% preparations of 11S globulins from soybeans and broad beans, containing sodium azide (0.02%), were stored in a dark place at room temperature in hermetically sealed vessels. Such storage provides invariability of thermodynamic and hydrodynamic characteristics of the preparations. Sedimentograms for the preparations of 11S globulins from sunflower seeds, peas, and oats were obtained immediately after their isolation. The concentration of globulins in the stock solutions was determined by drying to a constant weight at 105 ± 5 °C. The concentration of lysozyme in the solution was determined by spectrophotometry, assuming the extinction coefficient to be $E_{280}^{1\%} = 26.9$ (Khechinashvili, 1977). Solutions for sedimentation, viscosimetric, and calorimetric investigations were pre-

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