36. Papain Catalyzed Oligomerization of α-Amino Acids. Synthesis and Characterization of Water-Insoluble Oligomers of L-Methionine[1)]

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Summary

Water-insoluble oligomers were synthesized from L-methionine ethyl ester with papain as the catalyst. L-Oligomethionine was obtained in yields of 50% when synthesis was carried out in highly concentrated citrate buffer at pH 5.5. Yields of up to 85% were obtained when the enzymatic synthesis proceeded in distilled water at pH 6.5, the pH being strictly maintained. The insoluble polymer was converted to highly water-soluble sulfoxide and sulfone derivatives, which consist mainly of an octamer with low amounts of heptamer or hexamer. Most of the carboxyl terminals still contained the ethyl ester group, only a minor part being present in the free acid form. The potential of the enzymatic approach for the synthesis of optically pure and monodisperse oligomers of α-amino acids is discussed.

Introduction. – Proteases polymerize esters of α-amino acids to water-insoluble products. *Brenner et al.* [1] [2] demonstrated this for the chymotrypsin-catalyzed polymerization of the isopropyl esters of threonine and methionine. *Dannenberg & Smith* [3] reported the enzymatic synthesis of a phenylalanine oligomer by a protease from bovine lung. *Sluyterman & Wijdenes* [4] described the synthesis of an oligomer (8–9 residues long) of leucine methyl ester by papain. Recently, *Anderson & Luisi* [5] showed that papain catalyzed the oligomerization (6–7 residues) of tyrosine methyl ester. With the aim of exploring the potential of the enzymatic approach for the preparation of monodisperse oligomers of various α-amino acids, we undertook the study of the polymerization of methionine ethyl ester by papain. The difficult problem of analyzing water-insoluble peptides (generally poorly soluble in most organic solvents as well), was solved in our case by converting the water-insoluble polymer into a fully water soluble one by oxidation of the methionine to the sulfoxide or sulfone [6].

[1)] Keywords: *Enzymatic Peptide Synthesis, Papain, Methionine, Polymerization, α-Amino Acid Ester, Peptides, Sulfoxide, Sulfone.*

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Table 1. *Yields, total* N*-content and specific rotations of* L*-oligomethionine synthesized under different conditions.* In all cases, papain (2% by weight) was the catalyst used.

Conditions of synthesis	Total *N* isolated (%)	Yield (% of total *N*)	Specific rotation ($c = 2.5$, DMSO)		
			589 nm	578 nm	546 nm
1M sodium citrate pH 5.5	9.7	51	−12.7°	−13.6°	−15.2°
2M sodium acetate pH 5.5	9.9	16	−13.4°	−14.0°	−15.6°
2M sodium succinate pH 5.5	9.7	27	−12.8°	−12.4°	−13.6°
Dist. water; pH-stat pH 5.0	9.9	36			
Dist. water; pH-stat pH 5.5	10.2	65			
Dist. water; pH-stat pH 6.0	10.2	75			
Dist. water; pH-stat pH 6.5	10.4	85	−14.2°	−15.0°	−18.0°
Dist. water; pH-stat pH 7.0	10.2	61			

Result. – *The enzymatic polymerization.* The characteristics of the papain-induced synthesis of α-amino acids has been discussed [4–7]. From these studies, it appears that conditions for synthesis are somewhat more restricted than those for hydrolysis. In particular, satisfactory yields for the polymerization could be obtained only in a small pH range (between 5 and 6) and particularly when the reactions were carried out in citrate buffer [5] [6]. The presence of citrate buffer was instrumental in improving not only the overall yield, but also the homogeneity of the products. In the present work, carboxylic buffers other than citrate were tested *(Table 1)*. Both in sodium acetate and sodium succinate yields are appreciable, but not as high as in citrate. Most remarkably, conditions could also be found under which the reaction would proceed in distilled water (no salt or buffer added), provided that the pH is maintained strictly constant during reaction. This is surprising in view of the previous observation [5] that synthesis occurs more readily in concentrated than in diluted buffers, and in view of the observation that synthesis would not occur in 'auto-buffer' [5].

Table 2. *Data of elemental analysis*

Polymerization degree	Formula	Mol-wt.	Calc. %			
			C	H	N	
6	$C_{30}H_{56}N_6O_7S_6$	805.19	44.75	7.01	10.44	
7	$C_{35}H_{65}N_7O_8S_7$	936.39	44.89	7.00	10.47	
8	$C_{40}H_{74}N_8O_9S_8$	1067.58	45.00	6.99	10.50	
9	$C_{45}H_{92}N_9O_{10}S_9$	1198.79	45.09	6.98	10.52	
10	$C_{50}H_{101}N_{10}O_{12}S_{10}$	1329.98	45.16	6.97	10.53	
			Found %			
			C	H	N	ash
Prep. sodium citrate:	prior to ester hydrolysis		45.16	7.17	9.69	1.23
Prep. sodium citrate:	after ester hydrolysis		45.26	6.98	10.51	2.34
Prep. water pH 6.5:	prior to ester hydrolysis		45.22	7.59	10.21	–
Prep. water pH 6.5:	after ester hydrolysis		45.76	7.62	10.58	0.55

The highest yield in water was obtained at pH 6.5 *(Table 1)*. Total N-content and optical rotation of this product were higher than those from the products synthesized in buffer. As mentioned in the experimental part, the sulfoxide derivative of oligomethionine synthesized in citrate buffer showed some heterogeneity and this was also true for oligomethionine synthesized in water. Chromatography of the sulfoxide derivatives in *Sephadex* G-25 fine in formic acid showed that a major peak was followed by a minor peak containing about 20–25% of the total peptide product. Both esterified and free carboxyl species were present in the two peaks and according to electrophoretic evidence, the second peak corresponded to lower oligomers, most likely heptamer and hexamer.

Chemical characterization of L-*oligomethionine.* Elemental analysis indicated that the formulas of the higher oligomers were compatible with our experimental data. However, the differences in C-, H- and N-percentages between two successive members of the series are not sufficient to permit use of elemental analysis data for the estimation of the polymerization degree *(Table 2)*. From the calculated N-content of 10.50% for an octamer (after removal of the C-terminal ethyl ester

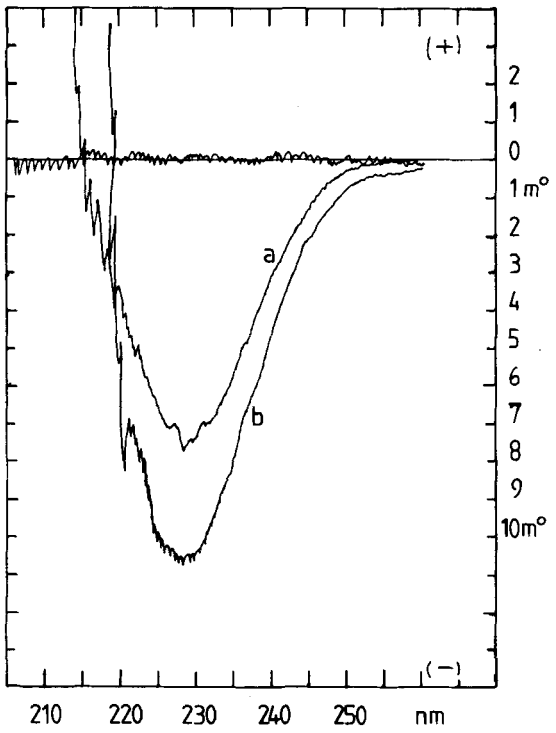

Fig. 1. *CD. spectra of* L-*oligomethionine (a) and heptamethionine methyl ester (b) in trifluoroethanol.* Concentrations: a = 0.50 mg/ml (approximately 0.47 mM assuming a molecular weight of 1068); b = 0.90 mg/ml (approx. 0.85 mM, molecular weight 1065 for the trifluoroacetate). Cell: 1 mm path length.

group), an average N-methionine conversion factor could be calculated: 100/10.5 = 9.52. This factor is useful to estimate the methionine content of our preparations of L-oligomethionine. Accordingly, a preparation with 9.7% of total N after hydrolysis of residual ethyl ester linkages should have a methionine content of 92.3%.

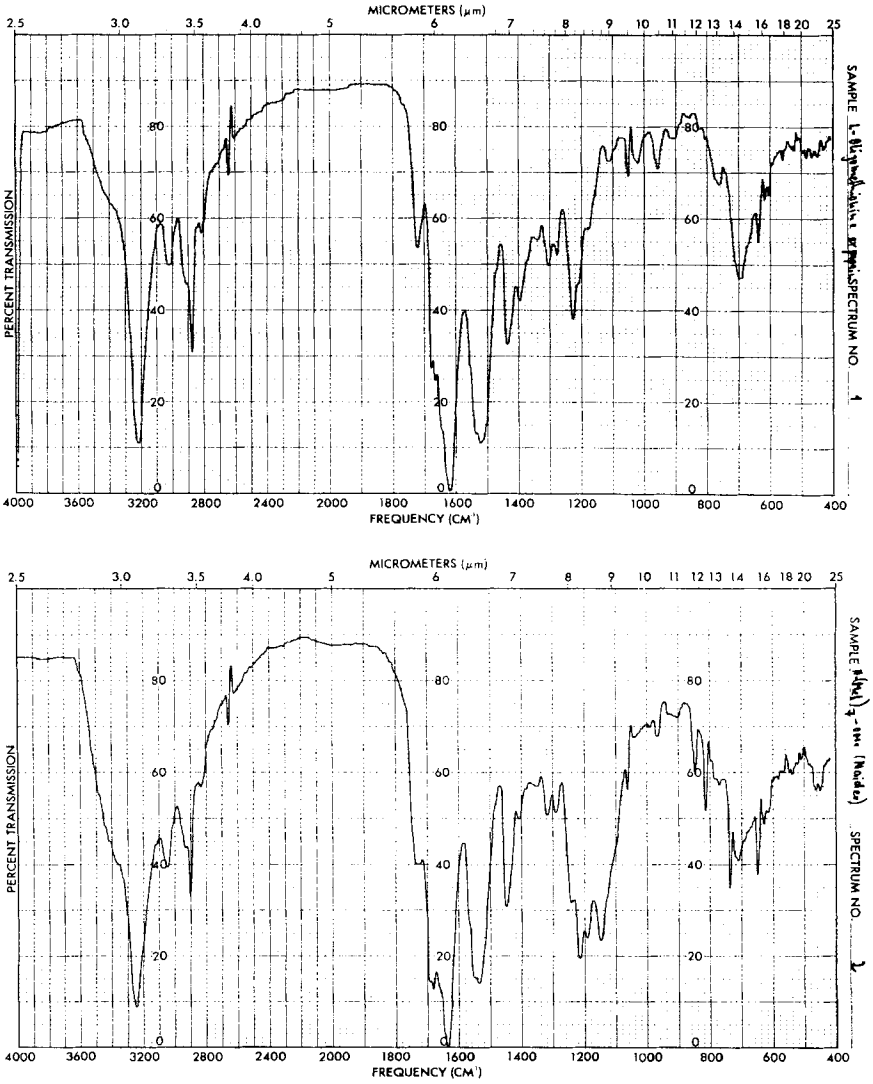

Fig. 2. *IR. spectra of* L-*oligomethionine (bottom) and heptamethionine methyl ester (top);* KBr-pill, peptide concentration 1%. Assignment of diagnostically important bands (cm^{-1}): 3230*s*, N–H stretching indicative of H-bonding in β-structures; 1710*w*, C=O vibration of C-terminal carboxyl- and esterified carboxyls; 1620*s*, C=O stretching vibration of the amide I band; 1520*s*, N–H deformation vibration of the amide II band; 700*m*, out of plane vibration of the amide V band.

Amino acid analysis of this preparation gave 870 μg per mg of methionine residue which is reasonably close to the expected peptide content. L-Oligomethionine prepared under different conditions always showed a weak levorotation of $[\alpha]_D^{25} = -12°$ to $-14°$ in dimethylsulfoxide *(Table 1)*. Its CD. spectrum in trifluoroethanol *(Fig. 1)* was characterized by two negative bands at 230 and 195 nm. This was essentially the same spectrum as that obtained with an authentic sample of heptamethionine methyl ester [8]. Thus, the band at 230 nm had a residue ellipticity $\theta = 1922$ deg cm^2 dmol^{-1} for the enzymatically synthesized oligomer and $\theta = 1842$ deg cm^2 dmol^{-1} for the synthetic heptamer. Interestingly, the BOC-protected heptamer gives an entirely different CD. spectrum with features indicating the presence of secondary structure. This shows that the nature of the amino terminal end has a profound influence on the conformation of the whole structure, which in turn demonstrates the importance of cooperative interactions in polypeptides. Similar results have been recently obtained by *Toniolo et al.* [9] in their study of the CD. properties of homooligo-L-methionines.

The IR. spectra of both the enzymatically synthesized oligomer and the synthetic heptamer were recorded in the solid state *(Fig. 2)*. The close similarity of the spectra is evident. However some minor differences might have diagnostic value. For example, the intensity of the 3230 cm^{-1} band ((N–H)-stretching) as compared with the shoulder at 3400 cm^{-1} is lower in the heptamer than in the enzymatic product. According to *Toniolo et al.* [10] this might be interpreted as due to H-bonding in a β-sheet pleated structure which is more significant at higher chain length. Further evidence of β-structures present in both compounds in their solid state comes from the position of the (C=O)-stretching band at 1620 cm^{-1} and from a decreased shoulder at 1660–1680 cm^{-1}.

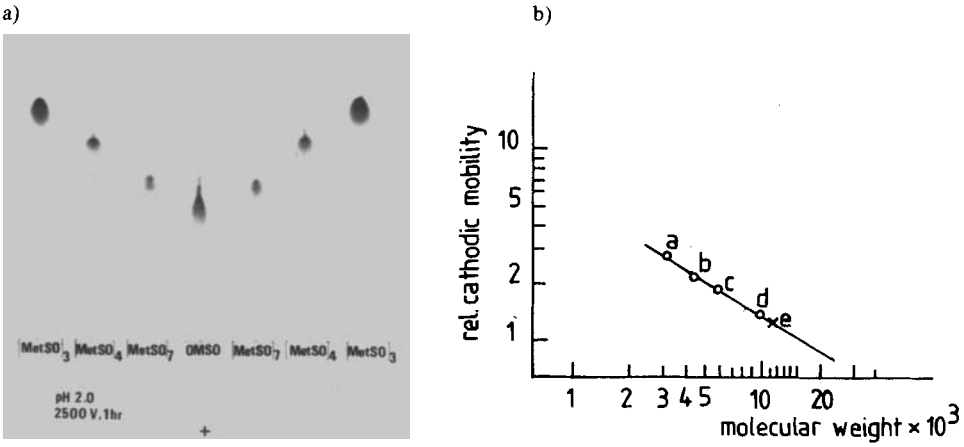

Fig. 3. a) *Relative cathodic mobilities of* L-*oligomethionine sulfoxide at pH 2.0;* 2500 Volt/1 h. Electrophoretic purity at this pH indicated the presence of a major species (octamer) and that of a heptamer or hexamer on low quantity. b) Log/log plot of the relative cathodic mobilities of several oligomers *vs.* their molecular weight. All peptides are sulfoxide derivatives. a = dimer, M 303.4; b = trimer, M 459.6; c = tetramer, M 606.8 and d = heptamer, M 1048.4. X = mobility of oligomethionine sulfoxide (main spot): corresponding M *ca.* 1200.

Table 3. *Estimation of the polymerization degree ($\bar{n}$) by the ninhydrin reaction.* A: % α-amino-N prior to acidic hydrolysis, B: % α-amino N following acidic hydrolysis. DH = degree of hydrolysis; preparation 1) was oxidized with DMSO/HCl/HOAc; 2) with H_2O_2 in 0.2N NaOH and 3) with H_2O_2 in presence of HOAc at pH 5.0.

Preparation	% total N	α-amino N:A	α-amino N:B	DH = A × 100/B	$\bar{n}$ = 100/DH
1) Sulfoxide	8.5	9.0	65.0	13.8	7.2
2) Sulfone	8.7	8.9	70.1	12.7	7.8
3) Sulfoxide	8.3	8.9	77.7	11.5	8.7

Polymerization degree of L-*oligomethionine.* The cathodic mobility of a homopolymer such as oligomethionine at pH 2.0 should be a function of its molecular weight, as oligomers of different chain length all have the same net charge. In order to have comparable standards, we prepared the sulfoxide derivatives of di-, tri-, and tetramethionine, and we converted heptamethionine methyl ester to the sulfoxide derivative. These standards were analyzed together with the sulfoxide derivative of L-oligomethionine. The main fraction of the enzymatically synthesized oligomer had a mobility distinctly below that of the heptamer *(Fig. 3a)*. Some minor components (25%) were also present which corresponded most likely to a heptamer or hexamer. From the double logarithmic plot [11] *(Fig. 3b)* an approximate molecular weight of 1200 could be deduced for the main fraction, which corresponds to an octamer. We have also attempted to estimate the molecular weight by end-group analysis. Meaningful results could only be obtained by using the water-soluble derivatives of the oligomer (several methods to quantify end-groups such as dinitrophenylation, dansylation or ninhydrin reaction gave unreproducible results with the water-insoluble polymer). We applied the ninhydrin reaction to the sulfoxide and sulfone derivatives and determined their α-amino N-content (with the ninhydrin reaction) prior to and after total acidic hydrolysis. With the aid of these data *(Table 3)* we established polymerization degrees ranging from 7.2–8.7 residues, depending on the derivative.

A more precise determination was obtained from NMR. data for a pure ethyl ester component. The preparation of the latter was not possible from L-oligomethionine as such, but it was readily achieved from the sulfoxide derivative. The spectrum of a completely esterified sulfoxide derivative is shown in *Figure 4*. Representative for the C-terminals of the peptide chains were the 3 protons of the methyl group of the ester which gave a clean triplet at 1.2 ppm. The ratio of side chain protons *vs.* C-terminal ester protons was this time 8.26, indicating an average polymerization degree of 8 residues. The ^{1}H-NMR. spectrum of H-$(Met)_n$OEt in (D_6) DMSO has been reported previously [6].

Discussion. – The papain-catalyzed reaction which leads to the methionine oligomers described in this work is a transesterification. In the case of papain, reactions of this type have already been investigated [4–6] [12]. In particular, *Glazer* [12] has shown that when glycylglycine pentylester was incubated at pH 5 in aqueous solution with papain, the reaction mixture contained glycylglycine, tetraglycine, tetraglycine pentylester, and traces of hexaglycine and higher polymers.

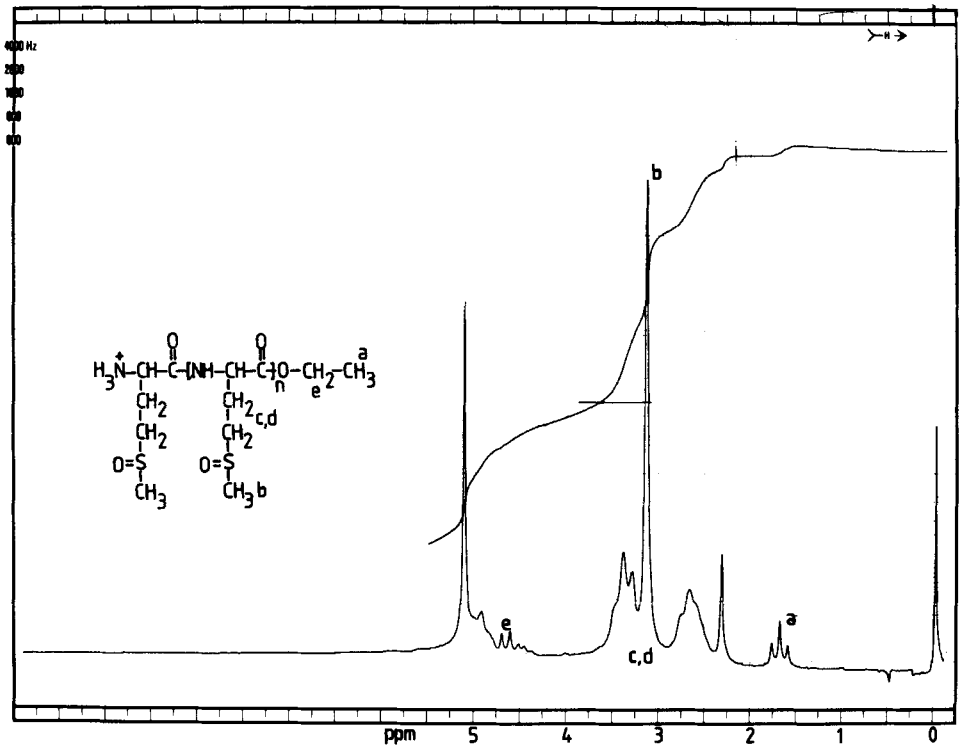

Fig. 4. *^1H-NMR. spectrum of* L-*oligomethionine sulfoxide (purified ethyl ester species) in D_2O;* 80 MHz, 36°. Polymerization degree ($\bar{n}$): $-CH_2-CH_3$ (3 H) = 2.5 units; 1 ester proton = 0.83 unit. $-CH_2-CH_2-S(O)-CH_3$ (7 H) = 48 units; 1 side chain proton = 6.86 units. $\bar{n} = 6.86/0.83 = 8.2$.

Also, the hydrolysis rate of the ester group was much larger than the hydrolysis of the peptide linkage (neither glycine nor triglycine was found in the reaction mixture). The major difference with the proposed mechanism [12] is that in our case the higher oligomers were insoluble and precipitated, the equilibrium being thus shifted towards a single species. Interestingly, simple amino acids esters are reported to be very poor substrates for transesterification [13] and methionine has never been reported as a typical substrate for papain [14]. Thus, in a sense, the good results obtained with the polymerization of the methionine ethyl ester are surprising.

Surprising, or at least difficult to rationalize, are also the conditions of the synthesis (in particular the fact that the best yields are obtained at two extreme conditions, high citrate buffer concentration, or distilled water, but not in diluted citrate buffer). Most probably, the explanation has to be sought in the fact that the product distribution and the yield are governed by subtle differences in solubility (*e.g.* between an octamer and the lower oligomers), and the external conditions (buffers, pH and salts in our case) can modify these small solubility differences of the various species to a different extent.

The main aim of this work was to establish whether, and to what extent, the enzymatic synthesis could be useful as a preparative tool for monodisperse oligomers of amino acids. We found the conditions under which the major component is an octamer, but a small fraction of lower oligomer is still present. Can the enzymatic synthesis be perfected to enable even higher homogeneity? We are at present investigating the influence of external parameters (mostly temperature and solvent) on the molecular weight distribution. Preliminary attempts to see whether the method can be modified so as to provide much higher oligomers, carried out with amino acids esterified with polyethylenglycol, were unsuccessful until now. The main drawback with this method is that when the starting amino acid is bound to a high molecular weight species, only very small molar concentrations can be reached in solution, which generally prevents polymerization. Attempts with solubilizing ester groups of intermediate length are in progress.

In conclusion, at the present stage, the enzymatic method in not yet perfectly set up to produce monodisperse oligomers with various polymerization degrees. However, the potential is so high and the advantages so patent (high yield of optically pure oligomers obtained in a single and very simple process) to justify further investigations.

The authors are indebted to Miss *B. Straub*, Technisch-Chemisches Laboratorium ETH-Z, for carrying out the CD. spectra. We are grateful to Dr. *F. Naider*, City University of New York, for the gift of a synthetic heptamer of methionine. We appreciated also the helpful comments of Dr. *C. Toniolo*, Biopolymer Research Center, Padova, on the IR. spectra of methionine oligomers.

Experimental Part

Materials. Commercial: papain, twice crystallized (*Sigma* Chemicals Co); methanesulfonic acid (*Pierce* Chemicals); di-, tri-, and tetramethionine (*Bachem* Liestal, Switzerland). L-Methionine ethyl ester hydrochloride was prepared in our laboratory, m.p. 80–83°, $[\alpha]_D^{25} = 30.8°$ ($c = 5\%$, H_2O). Heptamethionine methyl ester was the kind gift of Dr. *F. Naider*.

Analytical procedures. Total N was analyzed with an automatic Nitrogen Analyzer model 1300 of *Carlo Erba*, Milano. α-Amino N was analyzed by ninhydrin reaction [15]. Specific rotations were measured in spectral grade dimethylsulfoxide with a *Perkin Elmer* model 141 instrument. Amino acid analysis was performed according to standard procedures on a *Beckman* Multichrom B analyzer. For the hydrolysis of oxidized products, 4N methanesulfonic acid was used instead of 6N HCl in order to avoid reversion of methionine sulfoxide to methionine. High voltage paper electrophoresis was performed on *Savant* equipment and on *Whatman* 3 mm paper sheets. The pH 2.0 buffer was composed of formic acid (200 ml), glacial acetic acid (800 ml) and water (9 l). For the pH 6.5 buffer, 40 ml of glacial acetic acid and 1 l of pyridine (distilled over ninhydrin) were added to 9 l of water.

^{1}H-NMR. spectra were recorded on a *Varian* FT-80 MHz instrument. The solvent was (D_6) DMSO or D_2O. Circular dichroism spectra (CD.) were recorded on a *Jasco* J40AS spectrophotometer.

Elemental analysis was carried out both on the isolated products, and after hydrolysis of the ethyl ester linkages (4 h in 0.2N NaOH in ethanol) (see *Table 2*).

Enzymatic polymerization. – *Synthesis in carboxylate buffers.* The best results were achieved in 1M sodium citrate buffer, pH 5.5 (yields *ca.* 50%, *Table 1*). The concentration of the monomer, methionine ethyl ester, was usually 1.4M (300 mg of H-Met-OEt · HCl per ml of buffer). The temperature of the stirred monomer solution was 37°. Papain, dissolved in 0.1M L-cysteine, 10mM EDTA in water at pH 6.5, was added in 2 portions of 1% (by weight on the monomer) at time zero and after about 2 h of reaction at 37°. Since the reaction mixture completely gellified after about 2 h, this gel was homogenized with further citrate buffer and the suspension centrifuged. The sediment being kept, the supernatant solution was treated with the second portion of the enzyme and left at 37° overnight.

Synthesis in water. The monomer solution in distilled water (1.4M of the monomer) was taken to the desired pH with the use of the pH-stat; titrant was usually 2N NaOH. This time the reaction produced a milky suspension which did not form a gel. After about 1–2 h, the second portion of papain (1%) was added. The release of acid due to peptide synthesis ceased after about 4 h. Water-insoluble product was obtained within a wide range of pH, but the best yields ($\geqslant$ 80%) were achieved at pH 6.5 *(Table 1)*.

Product isolation. At the end of the reaction time, the mixture was diluted with 10 vol. of distilled water and briefly heated to 90° in order to destroy most of the enzyme activity. The suspension was then centrifuged (30 min at 10,000 rpm) and the supernatant solution discarded. The water-insoluble sediment was re-suspended in 200 ml of sterile distilled water and placed in an ultrafiltration cell. The cell, equipped with an *Amicon®* membrane type UM 10, was operated under 2.5 atm N_2 pressure to reduce the volume of the product suspension to $\frac{1}{20}$. Sterile distilled water was again added and the process repeated until at least 2 l of filtrate had been collected. At this stage most of the free monomer had been washed out, as indicated by ninhydrin reaction with the permeate. The content of the cell was now freeze-dried. A white powder with usually 9.5–10.5% total N-content was obtained by this procedure. Thus crude L-oligomethionine emerged from *Sephadex* G25 fine in dimethylsulfoxide as a single rather broad peak with a shoulder. The elution volume of the peak was about 1.3–1.4 void volumes. Higher resolution could not be achieved in this system. The sulfoxide derivative of the same compound could be chromatographed in 1% aqueous formic acid, when it gave a major, sharp and symmetric peak at 1.3 void volumes, immediately followed by a minor, slightly asymmetric peak at 1.6 void volumes.

The electrophoresis of several preparations of L-oligomethionine, and their corresponding sulfoxide derivatives at pH 6.5 (2000 V, 1.5 h), showed 2 main spots, with a relative mobility near zero and a cathodic mobility of 1. The latter spot corresponded to the ethyl ester species, as proved by saponification of the peptide in 0.2N NaOH in ethanol for 4 h at RT. After this treatment, the re-isolated product showed 1 spot in electrophoresis at pH 6.5 near the origin. The proportion of the free carboxyl form in different preparations of L-oligomethionine sulfoxide to varied between 20–40% of the total peptide material.

Soluble derivatives of L-oligomethionine. – *Sulfoxide derivative* (we used a procedure established by *Fontana & Savige* [16]). L-Oligomethionine (116 mg) was dissolved in a mixture of dimethylsulfoxide (140 µl), conc. HCl-solution (700 µl) and glacial acetic acid (1500 µl). The mixture was kept for 20 min at RT. and then treated with an excess of ice-cold water. This solution was evaporated to dryness, taken up in 1 ml of distilled water and applied to a *Sephadex* G-10 column in distilled water. The ninhydrin positive fractions were pooled and freeze-dried, when 100.5 mg of lyophilizate were obtained, total N-content 8.5%. Amino acids after hydrolysis in 4N methanesulfonic acid: methionine sulfoxide, 775 µg per mg; methionine sulfone, 0; methionine, 35 µg per mg.

Alternatively the sulfoxide derivative was prepared by using the H_2O_2-procedure, selective in acetic acid [17]. L-Oligomethionine (50 mg) was suspended in 1 ml of 30% H_2O_2 and a few drops of glacial acetic acid were added, giving a pH of about 5. After 20 min at RT., a few µg of catalase were added to destroy excess peroxide. The remaining peptide solution was freeze-dried to give 56 mg of lyophilizate with a total N-content of 8.3%. The hydrolysate (4N methanesulfonic acid, 24 h at 115° and 0.1 Torr) gave: methionine sulfoxide, 807 µg per mg; methionine sulfone, 0; methionine, 0.

Sulfone derivative. L-Oligomethionine (50 mg) was suspended in 10 ml of 0.2N NaOH and 1 ml of 30% H_2O_2 was added. After 20 min the reaction mixture was freeze-dried. The residue was redissolved in 1 ml of distilled water and applied to *Sephadex* G-10 in water. All peptide-containing fractions were pooled and freeze-dried to give 42 mg of a lyophilizate with 8.7% total N. Amino

acid analysis after hydrolysis in methanesulfonic acid: methionine sulfone, 679 µg per mg; methionine sulfoxide, 205 µg per mg; methionine, 0.

Isolation of a pure ethyl ester species. The sulfoxide derivative of oligomethionine (20 mg of the product with 8.3% N) was applied to a small *Dowex* column (*Dowex* I, X-2) equilibrated in 2M sodium acetate, pH 2.3. The esterified component of oligomethionine (12 mg) emerged unretarded from such a column, and appeared homogeneous by electrophoresis at pH 6.5. The lyophilizate was dissolved in D_2O and subjected to ^{1}H-NMR. analysis.

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