49. Papain-induced Oligomerization of α-Amino Acid Esters

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Summary[3])

Oligomers of leucine, methionine, phenylalanine, and tyrosine were prepared enzymatically (using papain) from the respective methyl esters, with yields ranging from 51–96%. The effect of pH, buffer and salt concentration, and addition of alcohol on the oligomerization of leucine methyl ester was examined. The limits and potentialities of the enzymatic reaction as a method for obtaining monodisperse oligomers are discussed.

Introduction[4]). – The last two years have witnessed a renewal of interest in enzyme-catalyzed synthesis of peptide bonds [1–5]. Usually syntheses are carried out by using soluble reagents which, upon coupling, produce an insoluble product – thus the equilibrium is shifted towards synthesis despite the unfavorable standard free energy change for synthesis in solution.

An interesting question is to what extent the enzymatic method lends itself to the preparation of poly-α-amino acids. This question is not new. As long ago as 1950 *Brenner et al.* found that α-chymotrypsin polymerizes the isopropyl esters of methionine, threonine, phenylalanine and tyrosine [6] [7]. *Fruton et al.* reported a short time later the (Cathepsin C)-catalyzed polymerization of various dipeptide amides [8] [9], the study of which was continued into the sixties [10–12]. They also found that ficin, as well as α-chymotrypsin, polymerizes methionine [13], and *Dannenberg & Smith* reported in 1955 that an enzyme isolated from bovine lung was capable of polymerizing several amino acid esters [14]. In a report by *Slyterman & Wijdenes*, the kinetics of papain-catalyzed polymerization of leucine methyl ester [15] was investigated. In all these studies, polymerization degrees ranging from 2 to 11 were reported, but few of the products have been isolated and thoroughly charac-

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[3]) Keywords: papain, enzymatic polymerization, leucine, methionine, phenylalanine, tyrosine, tryptophan, oligopeptides.

[4]) The following abbreviations are used in this paper: Z, benzyloxycarbonyl; BOC, *t*-butoxycarbonyl; Ac, acetyl; OMe, methyl ester; BuOH, butanol; HOAc, acetic acid; TLC., thin layer chromatography; MS., mass spectroscopy; NMR., nuclear magnetic resonance spectroscopy; UV., ultraviolet spectroscopy; Leu, leucine; Phe, phenylalanine; Met, methionine; Gly, glycine; Glu, glutamic acid; Tyr, tyrosine; Ala, alanine; Gln, glutamine; Trp, tryptophan.

terized. In general, in all those cited studies, emphasis was on the mechanism and kinetics, and much less on the utilization of the enzymatic reaction as a preparative synthetic procedure.

In this paper, we will re-examine some of the problems involved in enzyme-induced oligomerizations on a preparative scale, using the low-cost enzyme papain as the catalyst. In particular, we will investigate under which conditions a single and well characterizable product is obtained, and to what extent it is possible to modify the reaction conditions so as to modulate the polymerization degree.

Experimental Part. – *Materials.* H-Leu-OMe · HCl, H-Met-OMe · HCl, H-Tyr-OMe · HCl, BOC-Gly-OH, Z-Gly-OH, H-Phe-OMe · HCl, BOC-Leu-OH, Z-Met-OH, H-Trp-OMe · HCl and citric acid (used to make buffer) were all *puriss.* products purchased from *Fluka.* All reagents were checked chromatographically (*Merck* pre-coated Silica gel 60 F254, BuOH/HOAc/H_2O 3:1:1) and found to give a single spot. The papain used was *Fluka, purum.*

Methods. – *Instruments.* All melting points were done on a *Kofler* melting point apparatus (from *Reichert*). UV., CD., NMR., MS. and amino acid analyses were done on a *Beckman* Acta MV1, *Jasco* J-40AS, *WH*-90, *Hitachi Perkin-Elmer* RMU-6L and a *Bio Cal* BC201 (with Durum resin DC6A), respectively.

Synthetic procedure. Most of the syntheses were carried out by adding ~ 140 mg of the *N*-protected reactant and *ca.* 340 mg of the amino acid ester to be polymerized to 5 ml of 2.0M citric acid buffer (pH ~ 5.0), which contained ~ 30 mg enzyme. The solutions were then shaken mildly at room temperature for 1–3 days. In most cases a thick, gel-like precipitate had formed by this time. The precipitate was then filtered off (the filtrate being kept so as to collect a 2nd crop) and washed successively with two 2 ml portions of 5% Na_2CO_3-solution, two 2 ml portions of 1M HCl (except for non-protected products, *e.g.* H-$(Tyr)_n$-OMe) and finally with two 2 ml portions of water. The resulting products were dried under high vacuum and the yield calculated on the basis of this crude product (including the 2nd crop, if any). The crude products were then chromatographed (after dissolving in formic acid) and recrystallized in the appropiate solvent mixture.

Elemental analyses of the final products.

Z-Gly(Leu)$_5$-OCH$_3$ ($C_{41}H_{68}N_6O_9$): Calc. C 62.44, H 8.63, N 10.66%; Found C 61.93, H 8.64, N 10.36%.

Z-Gly(Met)$_4$-OCH$_3$ ($C_{31}H_{49}N_5O_8S_4$): Calc. C 49.80, H 6.56, N 9.37%; Found C 48.89, H 6.62, N 9.48%.

BOC-Gly(Leu)$_4$-OCH$_3$ ($C_{32}H_{59}N_5O_8$): Calc. C 59.91, H 9.20, N 10.92%; Found C 58.81, H 9.11, N 10.75%.

BOC-(Leu)$_4$-OCH$_3$ · 1H$_2$O ($C_{30}H_{58}N_4O_8$): Calc. C 59.80, H 9.63, N 9.30%; Found C 59.76, H 9.34, N 9.42%.

Z-Gln(Phe)$_3$-OCH$_3$ · 1H$_2$O ($C_{41}H_{47}N_5O_9$): Calc. C 65.34, H 6.24, N 9.30%; Found C 64.61, H 5.97, N 9.40%.

Z-(Met)$_9$-OCH$_3$ ($C_{54}H_{91}H_9O_{12}S_9$): Calc. C 48.18, H 6.77, N 9.37%; Found C 46.55, H 6.76, N 9.04%.

H-(Tyr)$_7$-OCH$_3$ · 5H$_2$O ($C_{64}H_{79}N_7O_{20}$): Calc. C 60.81, H 6.10, N 7.76%; Found C 60.86, H 5.97, N 8.03%.

Determination of the polymerization degree. – a) by UV.: (for Z$(Met)_n$OMe – see Results and Discussion) a small amount of sample was carefully weighed out and dissolved in formic acid. The absorbance was read from the 268 nm peak, using the absorbance at 277 nm as the baseline (because of UV. absorbing impurities giving rise to a slight tail from 227– ~ 300 nm which is not present in the control, Z-Met-OH). The ε used was 112.4 M^{-1} cm^{-1}.

b) By NMR.: In most cases the ratio of protecting group protons to the aliphatic protons of the repeating unit was taken. For Z as protecting group this means comparing the aromatic singlet at 7.3 ppm to either the CH_2CH signal at 1.7 ppm (for R$(Leu)_n$OMe) or to the combined $CH_2-CH_2+CH_3$ integrated signal from 2.2–2.8 ppm (for R$(Met)_n$ · OMe). In the case of Z-Gln$(Phe)_n$-OMe, where aromatic

protons are also present in the chain, integration of the methoxy signal (3.8 ppm) was compared to that of the α-carbonatom of the repeating unit (4.9 ppm). For BOC as protecting group the procedure was slightly more involved. Since the BOC signal in CF_3COOH comes at 1.5 ppm, that is together with the CH_2–CH signal of the repeating unit (Leu), it was necessary to first calculate the CH_2–CH intensity (on the basis of the clean CH_3(Leu) at 1.0 ppm) and compare the thus derived BOC signal to that of the clean CH_3 protons of the repeating unit. The fact that the BOC group may be cleaved under these conditions does not affect the results. For H-$(Tyr)_n$-OMe the methoxy signal was compared to that of the CH_2 protons.

c) By MS.: In cases where a clear molecular ion peak was not seen (*e.g.*, products I, V) the length of the chain was determined by the caracteristic decay of these *N*-protected peptides. That is, for Z-R$(R')_n$-OCH_3 (where R = Gly or Gln, and R' = Leu or Phe) cleavage was seen at every C=O bond (starting at the Z end). For BOC-R$(R')_n$-OMe (where R = Gly or Leu, and R' = Leu) the successive loss of each backbone group was clearly detectable (*e.g.*, for BOC-Gly$(Leu)_n$-OMe beginning with $(CH_3)_3$–C–O and continuing C=O, NH, CH_2, *etc.*).

d) By amino acid analysis (for product II – see *Table 2*): A sample of product was first hydrolyzed in 6M HCl for 16 h at 110° in a sealed tube which had been purged of oxygen. The contents were then roto-vapored to dryness and given for analysis. Only those amino acids expected from the reported composition of the product were detected.

Concerning the characterization of the products reported in *Table 2*, the following should be noted. The different methods used to determine the polymerization degree did not always give results which were in perfect agreement. The mass spectroscopy method is of course the most reliable, as it is unaffected by a small percentage of impurity – which on the contrary may strongly influence the other two spectroscopic methods. However, MS. could not be used in all cases, and elemental analysis is not sensitive enough to detect a small change in the polymerization degree.

Circular dichroic spectra of the products were found to be in fair agreement with literature values of similar products obtained by conventional methods (see footnote (d) in *Table 2*). Only in the case of products IV and VI was a value found that was significantly different from the literature, for which no explanation is as yet possible.

Results and discussion. – It is convenient to consider first the various factors which affect an enzymatic oligomerization. This can be done on the basis of a specific example, namely for the reaction Z-Gly-OH + H-Leu-OMe · HCl, which readily gives a product in high yield. Some of the data are summarized in *Table 1*. It can be seen from the first column that the yield was highest when buffer with an initial pH of ~5.6 was employed. One may recall that the optimum for the papain-catalyzed hydrolysis of peptide bonds is between pH 5–7 [19], while that for synthesis has been reported to be around pH 5 [20]. A study of this reaction at various buffer concentrations (second column of *Table 1*) revealed that the reaction was very sensitive to this parameter. The highest yield and cleanest product were obtained with the most concentrated buffer tested (2M citrate, pH 5.0). Not only was the yield in the 2M buffer experiment substantially higher than that with 1M, but the nature of the product was quite different as well. With concentrated buffer a single, clean spot was seen in TLC., while with 0.1M buffer a smear of products was obtained. This sensitivity of papain-catalyzed peptide bond formation to the citrate buffer concentration has been noted before [20]. We also observed that the reaction did not go at all in 'auto' buffer (reactants alone in solution, pH adjusted to desired value by addition of acid or base).

Also, for the reaction Z-Gln-OH + H-Leu-OMe (which, analogously to the Z-Gly-OH + H-Leu-OMe · HCl reaction, gives a product in high yield under standard conditions) no product was obtained with citrate/phosphate buffer. Also interesting are the results of the study on the effect of salt concentration (high concentra-

Table 1. *Influence of various factors on the yield*[a]) *of Z-Gly(Leu)$_n$-OMe*[b]) *synthesis*

Influence of pH			Influence of buffer conc.		Influence of [NaCl][d])		Effect of EtOH[e])	
pH[c]) initial	final	yield %	conc. (M)	yield %	conc. (M)	yield %	conc. (v:v)	yield %
3.80	3.56	0	0.10	9	0.00	76	0	76
4.41	3.89	15	0.25	32	0.014	75	10%	69
5.07	4.22	19	0.50	55	0.102	72	20%	40
5.64	4.42	22	0.75	74	1.00	65	30%	40
6.25	4.57	19	1.0	80	2.02	38	40%	10
			2.0	84	(sat.)	~0	50%	14

[a]) Referred to the crude product after washing procedure to eliminate unreacted starting material (based on a polymerization degree of n = 5).

[b]) Typical reactions were carried out by adding 135 mg Z-Gly-OH (6.45×10^{-4} mol), 340 mg H-Leu-OMe · HCl (1.87×10^{-3} mol) and 30 mg (20 mg for column 1) enzyme to 5 ml (6 ml and 3 ml for columns 1 and 2, respectively) of concentrated buffer (0.3M, 1M and 2M for columns 1, 3 and 4, respectively), pH 5.0, containing 1M NaCl unless otherwise specified.

[c]) The first value refers to the buffer (before addition of reagent), the second is the pH measured after three day's reaction.

[d]) The pH measured after 1 day's reaction was in all experiments in the range 4.2–4.3.

[e]) 1.0M citrate buffer containing no NaCl.

tions of salt have been advantageously used in the past by many workers engaged in enzymatic peptide synthesis [2] [1] [15] [3]. Surprisingly, we obtained the best results (third column) when no salt at all was added (in the presence, however, of 2M citrate buffer).

A single spot in TLC. was not always seen in the experiments reported in the first three columns of *Table 1*. For example, 2 spots with differing intensities were observed in the experiment described in the fourth entry of the second column, and two spots were present in most of the experiments of the third column. We have not analyzed the product distribution in all these various experiments since our main aim was to find conditions under which a single, clean product could be obtained. These experimental observations do indicate qualitatively, however, that the different chemical parameters also affect the heterogeneity of the oligomeric products. In several cases where two or three spots were detected, simple crystallization was effective in isolating a homogeneous sample.

The experiments described in the fourth column of *Table 1* (effect of ethanol concentration) were carried out in order to see whether a higher polymerization degree could be obtained in a solvent mixture having a solubility power for the products larger than that of a buffered water solution. Unfortunately, not only is the yield lower, but in addition no products having a polymerization degree higher than 5 (that found for reaction in water) could be detected. It is also interesting that 10% trifluoro-ethanol completely inhibits the reaction.

Let us consider now the question, which products can be obtained by the enzymatic polymerization procedure. Among the many experiments we have carried out, only those which permitted isolation of prevalently monodisperse oligomers are reported in *Table 2*. Only in a couple of cases could we find conditions for oligomerization starting from an amino acid methyl ester alone, namely for H-(Tyr)$_n$-OMe

Table 2. *Products obtained via enzymatic synthesis'*

	Product	n			Recrystallization solvent	M.p.	$[\theta]\cdot 10^{-4}$[c])[d]) [deg·cm^2], nm	Rf[e])	Yield[f])
		NMR.[a])	MS.[b])	other					
I	Z-Gly(Leu)$_n$-OCH$_3$	5.0	5		MeOH/H$_2$O[g])	247–252°	−8.3, 199	0.86	91 (n=5)
II	Z-Gly(Met)$_n$-OCH$_3$	3.9	-	4.4[h])	MeOH/H$_2$O 1:1	187–190°	−3.3, 197	0.81	96 (n=4)
III	BOC-Gly(Leu)$_n$-OCH$_3$	4.3	4		MeOH/H$_2$O 3:2	202–206°	−5.5, 197.5	0.73	82 (n=4)
IV	BOC-(Leu)$_n$-OCH$_3$	4.0	4		MeOH/H$_2$O 3:2	148–152°	−3.2, 200.5	0.78	79 (n=4)
V	Z-Gln(Phe)$_n$-OCH$_3$	3.4	3		DMF/H$_2$O 1:1	224–228°	−0.2, 235 +1.7, 216.5	0.73	56 (n=3)
VI	H-(Tyr)$_n$-OCH$_3$	6.7	-		MeOH/H$_2$O 1:1	dec. 210	+3500, 230 (per residue)	0.78	28 (n=7)[i])

[a]) In CD_3COOD for II, in CF_3COOH for all other products.

[b]) Mass spectroscopy could not be used for products II and VI.

[c]) All CD. work was done in trifluoroethanol at a concentration of ~0.1 mg/ml, except for VI, TFE, H_2O (83:17 *v/v*).

[d]) Literature values for related compounds: BOC-(Leu)$_4$-OCH$_3$ [16], −6.0 (198 nm); BOC-(Leu)$_5$-OCH$_3$ [16], −9.0 (199 nm); BOC-(Met)$_4$-OCH$_3$ [17], −4.6 (196 nm); BOC-(Phe)$_3$-OCH$_3$ [18], −0.10 (238 nm) and +2.3 (217 nm); BOC-(Tyr)$_7$-OCH$_3$ [26] + *ca.* 5000 (230 nm) per residue, in propanediol DMSO (99:1 *v/v*).

[e]) In BuOH/HOAc/H$_2$O 3:1:1, spots detected with iodine; Rf values are averages of 2 to 6 runs.

[f]) Referred to our standard conditions (see expt.) after three days and calculated on the basis of the n value given in parentheses. Product I was obtained using 10% methanol in the reaction mixture.

[g]) Dissolved in hot MeOH, then H_2O added dropwise to the hot solution until it became cloudy; the solution was then cooled in ice and the precipitate collected.

[h]) Amino acid analysis.

[i]) This compound makes up ~40% of the crude product, as determined by a preliminary chromatographic separation on Sephadex LH-20. The residue appears to be composed of higher molecular weight fraction(s).

(product VI of *Table 2*), H-(Trp)$_n$-OMe (not characterized) and H-(Met)$_n$-OMe. In all the other cases the enzyme needed a 'starter' in the form of Z-Gly-OH, BOC-Leu-OH, BOC-Gly-OH or Z-Gln-OH (a known starter [13]). The compound H-(Met)$_n$-OMe is obtained in very good yield (~90% by weight) but was not characterized, and therefore is not reported in *Table 2* (Z(Met)$_n$-OMe can be made as well, starting from Z-Met-OH + H-Met-OMe · HCl). Infact, fractional crystallization was not capable of resolving this product beyond two spots in TLC. The average polymerization degree for the 2-component product was ~9, as determined by UV. and NMR.). The enzymatic preparation of oligomers of the type H-(Met)$_n$-OMe has already been reported in the literature [6] [7].

Other reactions which were not worked up, but which gave product in fair to good yield, were Z-R-OH (R = Ala, Gln or Glu) (also Ac-Phe-OH) plus H-Met-OMe · HCl, and the reaction Z-Ala-OH (or Ac-Phe-OH) plus H-Leu-OMe · HCl. Some failures included attempted oligomerizations utilizing known starters together with the methyl esters of histidine, serine and alanine. When the starter Z-Gly-OH was added to H-Phe-OMe · HCl (which readily oligomerizes with the starter Z-Gln-OH, see *Table 2*) no product at all was obtained. Some amide substrates (H-Leu-NH_2 · HCl, H-Phe-NH_2 · HCl, and H-Phe-Gly-NH_2 · HCl), together with starters, were also tried (papain is known to be capable of catalyzing transamidation reactions [21–23] [15] without success. We did not get clean results when Z-Leu-OH was used as starter with its respective partner, H-Leu-OMe.

In conclusion it apears to us that the present data permit the following generalization to be made. The enzymatic coupling procedure enables the rapid, easy preparation of several oligopeptides. At the present state of the art it does not appear possible, however, to forsee which products can be obtained in this way, nor to modulate in an easy way the polymerization degree by changing the reaction conditions. It appears then that the method is valid only for the preparation of selected types of oligomers, lacking therefore the general validity of a general routine synthetic procedure. However, within these limits, the advantages of the enzymatic reaction should not be underestimated. In fact, products are obtained by simply mixing reactants at room temperature, and pure products in a high chemical yield can be obtained with simple washing or 1-2 recrystallizations. Furthermore, it is expected, as has been shown for other enzymatic reactions [3] that optically pure products are obtained even starting from optically impure products or racemates. Finally, there are some still unexplored possibilities in the method which may warrant further investigation - for example, whether the polymerization degree of the products could be increased by attaching the 'starter' to a water soluble polymer, *e.g.* polyethylene glycol, which is already in use in the 'liquid phase' synthesis of peptides [24].

More in general, our studies reveal some aspects which are very interesting from the enzymologist's point of view: for example, the fact that peptide synthesis proceeds - in contrast to hydrolysis - only in citrate buffer; or that certain reactions unexpectedly fail to give products; or that monodisperse products can be obtained, but only under particular conditions. This extraordinary specificity of the enzyme towards synthesis is certainly correlated with basic stereochemical properties of the active site, as well as with the kinetic and thermodynamic properties of the actual reactions. Elucidation of these effects may reveal a good deal about enzyme's mechanism of action and its general behaviour. At the present stage of investigation, however, no rationalization as to these effects appears possible.

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50. *ipso*-Substitution with Sodium-*N*-alkyl-(*p*-nitrobenzene)sulfonamide. A Novel Anionic Rearrangement

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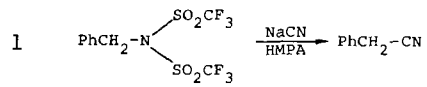
Summary

Reaction of *N*-benzyl-*N*,*N*-di-(*p*-nitrobenzene)sulfonamide (**3**) with NaCN/HMPA at 140° affords *N*-benzyl-*p*-nitroaniline (**4**). The same product is obtained upon heating of the sodium salt of *N*-benzyl-(*p*-nitrobenzene)sulfonamide (**5**). The transformation **5**→**4** is believed to proceed *via* an anionic episulfonyl compound **6**.

N-Benzyl-*N*,*N*-ditrifluoromethanesulfonamide (**1**) reacts with sodium cyanide in HMPA *via* nucleophilic displacement to give benzyl cyanide [1] *(Scheme 1).* The corresponding tosylamide **2** however, under the same conditions is converted to benzonitrile by formal two-fold elimination of *p*-toluene sulfinic acid [2] *(Scheme 2).*

Scheme 1



Scheme 2