BBA 66732

SIGMOIDAL PROGRESS CURVES IN THE POLYMERIZATION OF LEUCINE METHYL ESTER CATALYZED BY PAPAIN

L. A. Æ. SLUYTERMAN AND J. WIJDENES
Philips Research Laboratories, Eindhoven (The Netherlands)

(Received May 16th, 1972)

SUMMARY

The catalytic action of papain (EC 3.4.4.10) produces insoluble polyleucine peptides, eight to nine units long, from leucine methyl ester. This reaction is preceded by a pronounced induction period.

It can be concluded from the dependence of the reaction rate and of the length of the induction period upon monomer concentration and pH, that unprotonated leucine methyl ester is the reacting species.

The induction period is shortened, though not abolished, by the addition of dimer. Addition of a low concentration (0.02 mM) of trimer abolishes the induction period. At higher concentrations of added trimer a high initial rate is observed, owing to fast growth of trimer to octamer.

The phenomena can be explained by the combination of:

(i) a very slow dimerization rate and a high rate of chain growth from the trimer onwards;

(ii) the occurrence of "feedback" reactions, which produce new peptide esters of sufficient length to grow fast;

(iii) precipitation of the final product.

A highly simplified mathematical model confirms the explanation.

INTRODUCTION

For the examination of the activity of papain (EC 3.4.4.10) crystals a poor ester substrate was needed[1]. As such the methyl ester of leucine was tried, because leucine amide had been reported to be a poor substrate[2]. The ester was found to produce a rather unexpected phenomenon: a very pronounced induction period preceded the occurrence of polymerization. A description and an explanation of this phenomenon are presented in this paper.

EXPERIMENTAL

Materials

Mercuric papain was prepared by elution from an agarose mercurial column[3]. Leucylleucine, leucine methyl ester hydrochloride and leucine ethyl ester hydrochloride and dinitrofluorobenzene (DNFB) were purchased from Fluka (Switzerland), dinitrophenyl- (DNP-) leucine and $(Leu)_3$ from Mann Research Laboratories (U.S.A.), dithiothreitol from Calbiochem (U.S.A.) and trifluoroacetic acid from Schuchardt (Germany). $(Leu)_3$-OMe was prepared by dissolving 100 mg $(Leu)_3$ in 15 ml dry methanol containing 0.1 M HCl. After storage at room temperature overnight the methanol was evaporated by a current of dry N_2 at ambient temperature. The dry residue was treated once more in the same way and dried *in vacuo*. The esterification was checked by NMR (dissolved in hexadeutero-dimethyl sulphoxide). Only slight impurities were observed in thin-layer electrophoresis. $(Leu)_2$-OMe was prepared in the same way.

The methyl esters of glycine, alanine, valine, isoleucine and phenylalanine were prepared in methanol–HCl by conventional methods[4].

Methods

Automatic titrations were carried out in a pH stat of Radiometer (Denmark). An extra saltbridge was inserted between the reaction mixture and the calomel electrode.

The reaction mixture of 10 ml total volume generally contained 0.06 M Leu-OMe, 0.3 M KCl, 2.5 mM dithiothreitol, 1 mM EDTA and about 10^{-6} M papain.

Thin-layer electrophoresis was carried out on "Fertigfolie" Polygram Sil N-HR (Machery-Nagel, Germany), in 0.1 M formic acid *plus* 0.01 M KCl, for the analysis of $(Leu)_3$ hydrolysis, and in 1.5 vol. % pyridine in water, acidified to pH 5.5 with acetic acid, for the analysis of the polymerization mixtures. The semiquantitative determination of $(Leu)_2$-OMe concentration in the latter mixtures was carried out by spot comparison with standard mixtures.

Endgroup content

The precipitate, formed during a prolonged incubation of 50 ml reaction mixture, which had consumed 10 ml 0.1 M base, was thoroughly washed and suspended in 40 ml water. After addition of 800 mg $NaHCO_3$ and 80 ml of ethanol containing 1 vol. % of DNFB, the suspension was stirred overnight in the dark at room temperature. The precipitate was washed with 66 vol. % ethanol and dried *in vacuo*; the yield was 60 mg. Of this material 3.74 mg were dissolved in 8 ml trifluoroacetic acid and proved to give an absorbance of 5.90 at 360 nm.

DNP-leucine, dissolved in the same solvent, exhibited a molar extinction of 14.000. From these data a degree of polymerization of $N = 8.1$ can be calculated. Other similar determinations gave values of 8.4, 8.5 and 8.7.

RESULTS

On incubation of Leu-OMe and papain in the reaction vessel of a pH stat two phenomena were observed:

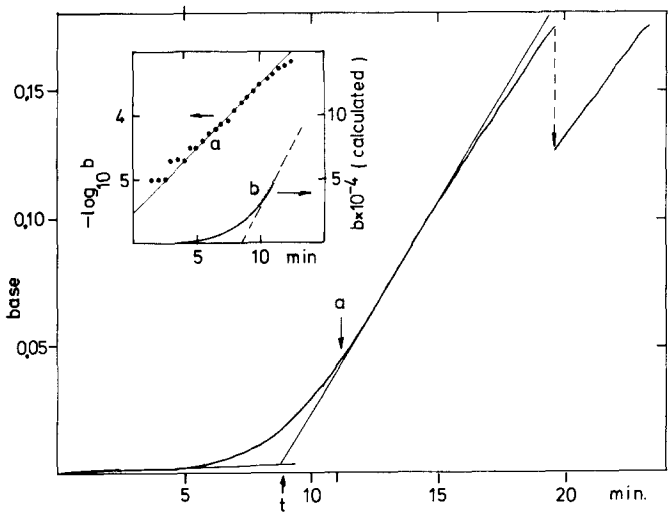

Fig. 1. Example of a progress curve of the polymerization of Leu-OMe. Conditions: 0.06 M Leu-OMe (pH 6.0, 25 °C), 2.4 μM papain, 0.3 M KCl, 1 mM EDTA, and 2.5 mM dithiothreitol. The ordinate indicates ml 0.1 M NaOH per 10 ml reaction mixture. The arrows indicate the induction time (t) and the onset of turbidity (a). Inset: a. Semilogarithmic plot of base consumption *vs* time. b. Calculated curve of base consumption *vs* time according to Eqn 11. The parameters were those from the slope and intercept of Curve a.

(1) On addition of papain, hardly any reaction was observed at first (Fig. 1). After an induction period the rate increased to a certain constant maximum rate, which slowly decreased again.

(2) Gradually a turbidity developed caused by insoluble material. With a narrow shaft of light through the flat bottom of the reaction vessel and black paper behind the vessel, the appearance of the turbidity could be observed. It coincided with the beginning of the linear part of the progress curve, indicated by Arrow a in Fig. 1.

The identity of the insoluble material will be dealt with first. It proved to be highly swollen material, rather resistant to 6 M HCl at 110 °C. After 5 days of hydrolysis most of the material had dissolved and appeared to consist entirely of leucine, as judged from thin-layer chromatography. The insoluble material therefore must have been polyleucine, a compound known to be insoluble in water.

The degree of polymerization was estimated by means of endgroup determination as described in the experimental part. The peptide chain proved to be 8–9 units long in the four cases examined.

The observation of polymerization in spite of the ordinary hydrolysis, indicates that four reactions may occur: transfer reaction with ester groups as donors; transfer reaction with peptide groups as donors; ester hydrolysis and peptide hydrolysis. In the neutral pH range ester hydrolysis and transfer reactions with ester groups as donors:

$$2\ H_3^+N\text{–}R\text{–}COOCH_3 \rightarrow H_3^+N\text{–}R\text{–}CO\text{–}NH\text{–}R\text{–}COOCH_3 + CH_3OH + H^+ \qquad (1)$$

consume base, whereas reactions involving peptide bonds do not do so.

Let us now examine the induction period, the length of which is defined as the time of intersection of the tangents shown in Fig. 1. It decreases with increasing pa-

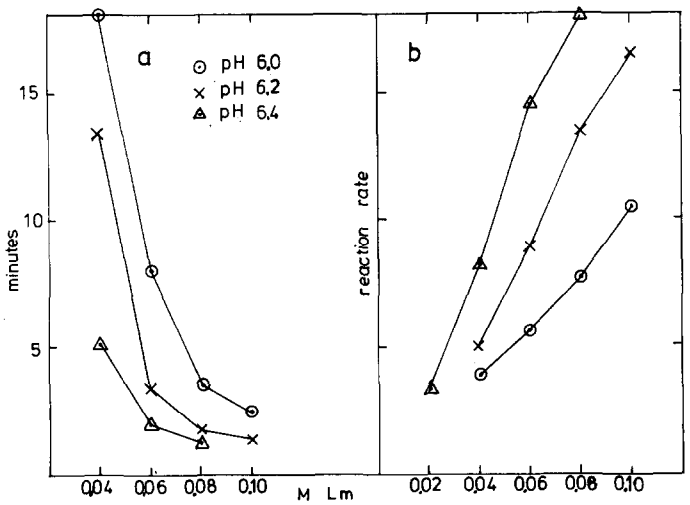

Fig. 2. Effect of Leu-OMe (Lm) concentration and pH upon (a) the induction period, (b) maximum rate (in arbitrary units).

pain concentration (not shown), increasing Leu-OMe concentration and increasing pH (Fig. 2). The induction period was totally absent when the papain was added as a suspension of crystals made insoluble by cross linking with glutaraldehyde[1].

The maximum rate increases with increasing papain concentration (not shown), increasing Leu-OMe concentration and increasing pH (Fig. 2).

In Fig. 2 the abscissa indicates the total concentration of Leu-OMe, which is actually composed of two species: those having a protonated and those having a free

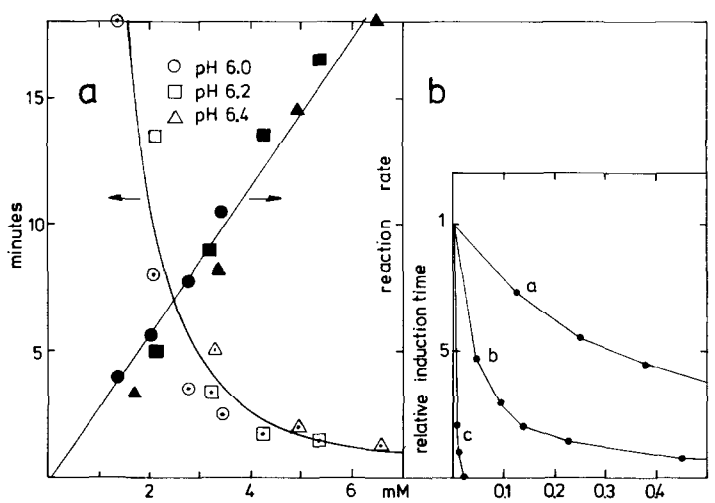

Fig. 3. a. Effect of concentration of nonionized Leu-OMe upon the induction period (open symbols) and the maximum rate (closed symbols). The former curve is drawn according to Eqn 2. b. Effect of $(Leu)_2$-OMe (Curve a), $(Leu)_3$ (Curve b) and $(Leu)_3$-OMe (Curve c) upon the induction time.

amino group. In order to test which species is actually involved in the reactions, the concentrations of the unprotonated species were calculated at each pH, utilizing the conventional ionization equation and a pK of 7.45 (derived by potentiometric titration). When the data of Fig. 2 are replotted as induction time and maximum rate *versus* concentration of unprotonated Leu-OMe, the results of Fig. 3a are obtained. Both quantities now gather approximately around one curve each, indicating their independence of pH. Hence, the unprotonated species serve both as donor and as acceptor in the polymerization reaction.

The curve in Fig. 3a is drawn according to the equation:

$$1/t = A[\text{Leu-OMe}]^2 \qquad (2)$$

where t denotes the induction time. Hence, as a first approximation, the induction time is governed by a reaction which is second order in Leu-OMe concentration (*cf.* Eqn 10 in Appendix).

The induction period suggests that the first steps of the polymerization occur at a considerably lower rate than the later steps. This was tested by adding small amounts of peptide or peptide esters to the reaction mixture. $(Leu)_2$-OMe was capable of decreasing the induction period (Fig. 3b) and of increasing the maximum rate. But even a concentration as high as 5 mM was unable to abolish the induction period entirely, whereas an ordinary reaction mixture after the induction period contained no more than about 0.3 mM $(Leu)_2$-OMe, as detected by thin-layer electrophoresis. $(Leu)_3$ was more effective than $(Leu)_2$-OMe but not effective enough either.

On the other hand, $(Leu)_3$-OMe proved to be very effective: a concentration as low as 0.02 mM abolished the induction period entirely (Fig. 4). Higher concentrations of $(Leu)_2$-OMe even caused a very fast reaction phase, after which the rate returned to the normal maximum rate. This is a direct demonstration that $(Leu)_3$-OMe is capable of growing very fast. The base consumption of this fast phase, as derived from

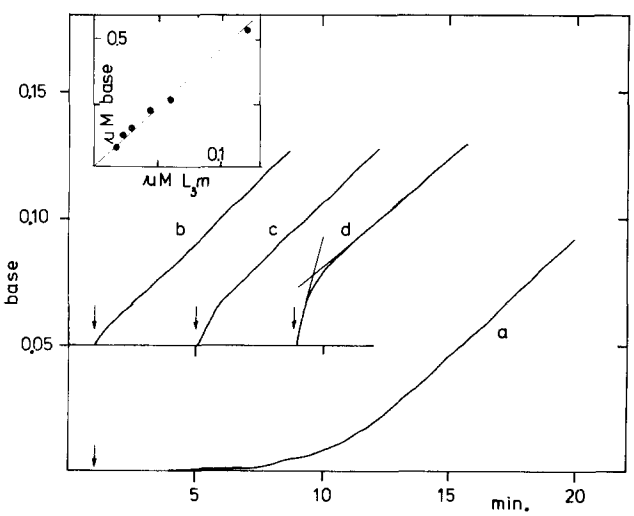

Fig. 4. Effect of $(Leu)_3$-OMe upon the progress curve of Leu-OMe (0.06 M) conversion at pH 6.0. The arrows indicate the moment of addition of papain. Curve a, no added $(Leu)_3$-OMe; curves b, c and d, 0.018, 0.030 and 0.06 mM $(Leu)_3$-OMe (L_3m) respectively. Inset: Base consumption of the fast phase as a function of $(Leu)_3$-OMe concentration.

the tangents drawn as indicated in Fig. 4, Curve d, is proportional to the concentration of $(Leu)_3$-OMe (Fig. 4, inset). According to the slope of the line, each mole of $(Leu)_3$-OMe liberates 4.7 protons.

These data show that the following reactions occur at the indicated rates

$$2\ \text{Leu-OMe} \xrightarrow{\text{very slow}} (Leu)_2\text{-OMe} + CH_3OH \tag{3}$$

$$(Leu)_2\text{-OMe} + \text{Leu-OMe} \xrightarrow{\text{slow}} (Leu)_3\text{-OMe} + CH_3OH \tag{4}$$

$$(Leu)_3\text{-OMe} + \text{Leu-OMe} \xrightarrow{\text{fast}} (Leu)_4\text{-OMe} + CH_3OH \tag{5}$$

$$(Leu)_8\text{-OMe} + \text{Leu-OMe} \xrightarrow{\text{fast}} (Leu)_9\text{-OMe} + CH_3OH \tag{6}$$

When the polymer chain has grown to a certain extent and the concentration of such a polymer is high enough the polypeptide will precipitate and will be withdrawn from further reactions.

The slowness of Reaction 3 is apparent from the exceedingly low rate at the start of the reaction. The medium rate of Reaction 4 is apparent from the enhancing effect of $(Leu)_2$-OMe on the maximum velocity. The rate of Reactions 5 and 6 is apparent from the fast phase of the reactions with added $(Leu)_3$-OMe.

Reactions 3–6 still cannot explain the induction period, because Reaction 3, being the first and slowest step, limits the rates of the succeeding reactions. Hence there must be a further source of peptide esters of at least three units long. These can be produced by transfer reactions like:

$$\text{Leu–Leu–Leu–Leu–Leu-OMe} + \text{Leu-OMe} \rightarrow 2\ \text{Leu–Leu–Leu-OMe} \tag{7}$$

The probability of such a reaction will increase with increasing chain length until the polypeptide precipitates. In this manner one growing chain can be converted into two growing chains, two growing chains into four, *etc.* This kind of reaction provides the autocatalysis required to explain the induction period. The reaction rate increases until a steady state is reached when the rate of genesis of new growing peptides equals the rate of precipitation of grown chains.

The occurence of reactions like reaction 7 is already indicated by the effect of $(Leu)_3$ upon the induction period (Fig. 3b), indicating the reaction:

$$(Leu)_3 + \text{Leu-OMe} \rightarrow (Leu)_3\text{-OMe} + \text{Leu}$$

Furthermore the susceptibility of the leucylleucine bond was substantiated by the hydrolysis of $(Leu)_3$ into $(Leu)_2$ and leucine catalized by papain.

Since no marked acceleration during the fast phase was observed on close inspection of curves like Fig. 4d, Reaction 7 does not occur to a great extent during this short period. Hence the approximately five protons liberated per $(Leu)_3$-OMe molecule during the fast phase indicate an increase in chain length from 3 to 8 units. This is already quite near to the length of 8–9 units reported above.

Polymerization of other esters

The following compounds, at a concentration of 0.06 M, were tested at pH 6.0 for their ability to polymerize: the ethyl ester of leucine and the methyl esters of

glycine, alanine, valine, isoleucine and phenylalanine. Leucine ethyl ester was polymerized after an induction period 5 times longer than that observed with the methyl ester. Of the methyl esters only the ester of phenylalanine yielded a polymer after standing overnight. In the latter case $(Leu)_3$-OMe (0.06 mM) gave a considerable rate enhancement.

DISCUSSION

Polymerization of amino acid esters catalyzed by proteolytic enzymes has been reported before[5]. Therefore, it is not surprising that papain is capable of doing likewise. On the other hand, as far as the authors are aware, the sigmoidicity of the progress curves has not been reported before.

From the existing data it is possible to make a rough estimate of the ratio of the rates of the first step and the third step. These rates are:

$$v_1 = k_1[\text{Leu-OMe}]^2 \text{ and } v_3 = k_3[\text{Leu-OMe}]\,[(\text{Leu})_3\text{-OMe}]$$

The slope of the initial part of curve a of Fig. 4, about 0.01, equals v_1 (*cf.* Eqn 10 in Appendix).

The slope of the tangent of the fast phase of Curve d of Fig. 4, which indicates the rate of the five steps from $(Leu)_3$-OMe to $(Leu)_8$-OMe, is 5.0. The slope per step, therefore, is 1.0. Hence, the ratio of the rates is 0.01.

Insertion of 60 mM for [Leu-OMe] and 0.06 mM for [Leu_3-OMe] (see caption to Fig. 4) yields $k_1/k_3 = 10^{-5}$.

Berger and Schechter[6] have shown that the rate of hydrolysis of peptides increases with increasing chain length. The present observation, that Reaction 3 is slower than Reaction 4 and that Reaction 4 is slower than the next reactions, fits into their observations.

According to Reactions 3–6 the peptide chain grows by one acceptor unit at a time. Of course short peptide esters could also act as acceptors (*cf.* ref. 7).

Indeed, if for instance $(Leu)_3$-OMe alone at a concentration of 0.6 mM is subjected to papain action, a trace of polymer is formed (besides ester hydrolysis as the dominant reaction), indicating that $(Leu)_3$-OMe too can be an acceptor. However, in the steady-state phase of the reaction of 60 mM Leu-OMe the concentration of $(Leu)_3$-OMe, as judged from Curve b of Fig. 4, is no more than 0.02 mM. Therefore the action of $(Leu)_3$-OMe as acceptor under the latter conditions is negligible and the chain growth by one unit at a time is the main reaction.

Because of the many reactions involved, an accurate mathematical treatment of the data is not feasible. However, even a highly simplified model (see Appendix) is capable of explaining the phenomena.

It has been mentioned above that no induction period is observed in the presence of crystals of papain. The explanation for this effect is quite simple: a molecule of $(Leu)_2$-OMe, once generated inside a crystal, has to pass a very large number of enzyme molecules before leaving the crystal and therefore has an equally large number of opportunities to react further to its final size. Therefore it is the logical consequence of a very high local enzyme concentration.

APPENDIX

O. ELGERSMA AND L. A. Æ. SLUYTERMAN

The properties of the reaction system preceding the steady state, can be understood from the following highly simplified scheme:

$$M \xrightarrow{k_1'[M]} M_2 \xrightarrow{k_2'[M]} M_3 \qquad (8)$$
$$\uparrow\uparrow \underline{k_0'[M]}$$

in which k_1' represents the slow steps of Reactions 3 and 4, k_2' the fast steps of Reactions 5 and 6 and k_0' the feedback reactions like Reaction 7, which produce new starting points (two moles of M_2 for each mole of converted M_3) for the fast reactions.

The differential equations for this scheme are:

$$\frac{d[M_2]}{dt} = k_1'[M]^2 + 2\,k_0'[M]\,[M_3] - k_2'[M]\,[M_2]$$

$$\frac{d[M_3]}{dt} = k_2'[M]\,[M_2] - k_0'[M]\,[M_3]$$

Since the reactions k_1' and k_2' and not reaction k_0' consume base, the base consumption is given by:

$$\frac{db}{dt} = k_1'[M]^2 + k_2'[M]\,[M_2]$$

During the limited period of the runs, the decrease in $[M]$ can be neglected. Therefore $k_1'[M]$, $k_2'[M]$ and $k_3'[M]$ can be replaced by k_1, k_2 and k_0, respectively. Solution of this set of differential equations then yields:

$$b = \frac{k_1[M]k_2}{w_1 - w_2}\left[\frac{w_1 + k_0}{{w_1}^2}(e^{w_1 t} - 1) - \frac{w_2 + k_0}{{w_2}^2}(e^{w_2 t} - 1)\right] \qquad (9)$$

with

$$w_1 = \tfrac{1}{2}\left\{\sqrt{(k_0 + k_2)^2 + 4k_0k_2} - (k_0 + k_2)\right\}$$

$$w_2 = -\tfrac{1}{2}\left\{\sqrt{(k_0 + k_2)^2 + 4k_0k_2} + (k_0 + k_2)\right\}$$

For short reaction times e^{wt} can be approximated by $(1 + wt)$. Then Eqn 9 reduces to:

$$b = k_1[M]t = k_1'[M]^2t \qquad (10)$$

Detailed analysis shows that the term with $e^{w_2 t}$ can be neglected when $e^{w_1 t} > 2$. This simplifies Eqn 9 into:

$$b = \frac{k_1[M]k_2}{w_1 - w_2}\,\frac{w_1 + k_0}{{w_1}^2}\left(e^{w_1 t} - 1\right) = b_0\left(e^{w_1 t} - 1\right) \qquad (11)$$

In the interval in which $e^{w_1 t} >> 1$ a plot of log b *vs* t should yield a straight line. Such a plot has been made of the curve of Fig. 1, resulting in Fig. 1, inset, curve a. A straight line is indeed obtained, which deviates from linearity after 11–12 min, when the concentration of M_3 no longer increases, because the solubility of M_3 is exceeded and the polymer precipitates. Then the steady state is reached.

From the slope of the line and the intercept of the ordinate the values of $w_1 = 0.455$ and of $b_0 = 3.25 \cdot 10^{-6}$, respectively, can be calculated. Insertion of these values into Eqn 11 yields Curve b of Fig. 1 inset. This curve is a good approximation of the curve of Fig. 1, exhibiting a very pronounced induction time of the proper length.

REFERENCES

1 L. A. Æ. Sluyterman and M. J. M. de Graaf, *Biochim. Biophys. Acta*, 171 (1969) 277.
2 J. R. Kimmel and E. L. Smith, *Adv. Enzymol.*, 19 (1957) 267.
3 L. A. Æ. Sluyterman and J. Wijdenes, *Biochim. Biophys. Acta*, 200 (1970) 593.
4 R. L. M. Synge, *Biochem. J.*, 42 (1948) 103.
5 E. Katchalski and M. Sela, *Adv. Protein. Chem.*, 13 (1958) 369.
6 A. Berger and I. Schechter, *Phil. Trans. R. Soc. Lond. Ser. B*, 249 (1970) 249.
7 A. N. Glazer, *J. Biol. Chem.*, 241 (1966) 3811.