Tetrahedron Vol. 44, No. 12, pp. 3633 to 3637, 1988
Printed in Great Britain

0040-4020/88 $3.00+.00

YIELD OPTIMIZATION IN THE KINETICALLY CONTROLLED ENZYMIC PEPTIDE SYNTHESIS

E.K.BRATOVANOVA, I.B.STOINEVA and D.D.PETKOV

Laboratory of Biocatalysis, Institute of Organic Chemistry,
Bulgarian Academy of Sciences, 1040 Sofia, Bulgaria

(*Received in UK* 13 *January* 1988)

Abstract - The yield and its time-dependence in acylenzyme mechanism-based enzymic peptide synthesis are controlled by the proteinase kinetic specificity. The maximum yield is limited by a non-equilibrium constant K_{max}. Both K_{max} and the time, t_{max}, taken to attain the maximum yield, are directly related to the enzyme kinetic parameters. These relationships allow kinetic determination of yield optimization in kinetically controlled enzymic peptide synthesis.

In view of the chemical and chiral purity of the final product, recombinant-DNA peptide synthesis and enzymic peptide synthesis provide the most efficient synthetic routes to bioactive piptides. The second approach is best applied as kinetically controlled enzymic peptide synthesis[1-12]. Yield and its time-dependence are the main concerns in this new synthetic methodology. They are closely related to the proteinase mechanism and kinetic specificity. Thus, the kinetically controlled approach is based on the formation of an intermediate (acylenzyme) during the proteinase catalysis[3,13]. The optimal concentration of the amine component for a successful preparative synthesis is determined by the enzyme nucleophile specificity[14]. Recent theoretical analysis[15] predicted a dependence of the kinetically controlled maximum yield on the ratio of the specific constants for enzymic hydrolysis of the acyl donor and the peptide formed.

We report experimental data on the relationships between the yield-determining factors in kinetically controlled enzymic peptide synthesis and the kinetic parameters of aminolysis/hydrolysis reactions, catalysed by α-chymotrypsin and alkaline mesentericopeptidase, a proteinase closely related to subtilisin BPN'[16]. The results seem to demonstrate the usefulness of kinetic analysis for yield optimization.

RESULTS AND DISCUSSION

The kinetically controlled enzymic synthesis of a peptide RCONHR" from an N-blocked amino acid or peptide ester RCOOR' and a C-blocked amino acid or peptide $NH_2R"$ conforms to the basic acylenzyme mechanism[17]:

$$\mathrm{RCOOR' + E \underset{k_{-2}[R'OH]}{\overset{k_{+2}/K_s}{\rightleftharpoons}} RCOE \underset{k_{-4}/K_p}{\overset{k_{+4}[NH_2R"]}{\rightleftharpoons}} RCONHR" + E} \qquad 1.$$

$$\mathrm{RCOE \xrightarrow{k'_{+3}[H_2O]} RCOO^- + E}$$

where RCOE is the acylenzyme and the meaning of the kinetic constants follows from the scheme. When the rate of RCONHR" synthesis (v_s) equals the rate of its hydrolysis (v_h):

$$v_s = k_{+4}[RCOE][NH_2R"]_{max} = (k_{-4}/K_p)[RCONHR"]_{max} = v_h \qquad 2.$$

a kinetically controlled maximum of the peptide concentration $[RCONHR"]_{max}$ is observed (Fig.1). Since $[R'OH] << [H_2O]$, then $k_{-2}[R'OH] << k'_{+3}[H_2O]$, which allows the following expression to be obtained for [RCOE] on the steady-state assumption with respect to the acylenzyme:

$$[RCOE] = \frac{(k_2/K_s)[RCOOR']_{max}[E] + (k_{-4}/K_p)[RCONHR"]_{max}[E]}{k'_{+3}[H_2O] + k_{+4}[NH_2R"]_{max}} \qquad 3.$$

Substitution of this equation into 2. affords the expression for the kinetically

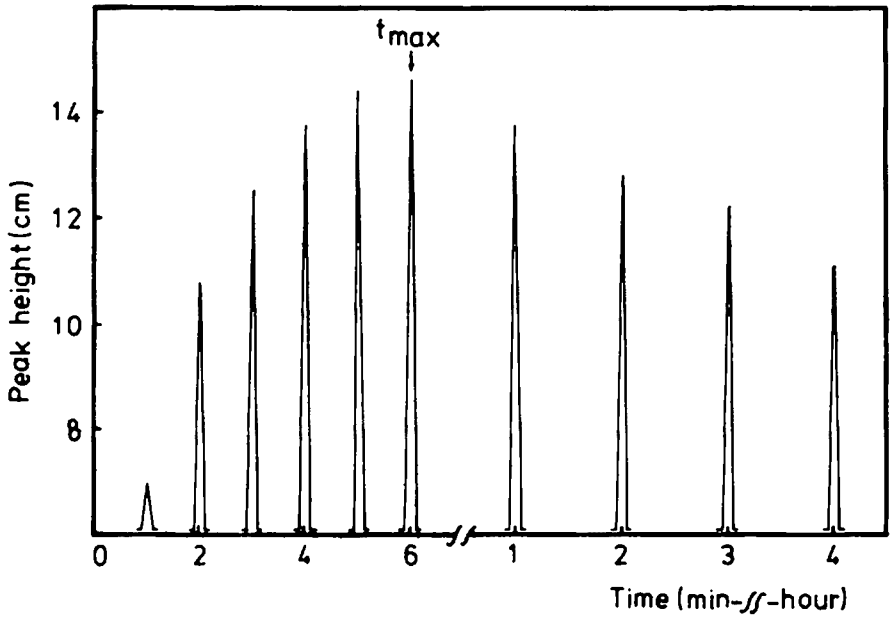

Figure: Time dependence of HPLC peak height of Ac-Gly-Phe(NO_2)-Gly-Leu-OH during the aminolysis of Ac-Gly-Phe(NO_2)-OMe (2 mM) by H-Gly-Leu-OH (82.5 mM), catalysed by alkaline mesentericopeptidase (0.05 μM).

controlled maximum concentration:

$$[RCONHR"]_{max} = \frac{(k_{+2}/K_s)}{(k_{-4}/K_p)} \frac{k_{+4}}{k'_{+3}} \frac{[NH_2R"]_{max}}{[H_2O]} [RCOOR']_{max} \qquad 4.$$

The latter may be easily rearranged into the following equation

$$K_{max} = \frac{\beta}{\alpha} \qquad 5.$$

where $\alpha = (k_{-4}/K_p)/(k_{+2}/K_s)$ is the specific constant ratio[15b] for the enzymic hydrolysis of RCONHR" and RCOOR', $\beta = k_{+4}/k'_{+3}$ is the aminolysis/hydrolysis ratio[14], and

$$K_{max} = \frac{[RCONHR"]_{max}[H_2O]}{[RCOOR']_{max}[NH_2R"]_{max}} \qquad 6.$$

We define K_{max} as a non-equilibrium constant of the kinetically controlled enzymic peptide synthesis. This maximum yield parameter limits the maximum non-

equilibrium amount of the peptide $[RCONHR"]_{max}$, accumulated during the synthesis, as an equilibrium constant K_e limits the yield in reactions under thermodynamic control. As follows from eq.5, K_{max} is a function of the proteinase kinetic specificity only.

The values of α obtained for chymotrypsin and alkaline mesentericopeptidase hydrolysis of acetyl-$(Gly)_n$-$Phe(NO_2)$-OMe and acetyl-$(Gly)_n$-$Phe(NO_2)$-Gly-Leu-OH (n = 0,1,2) are presented in the Table. The values of β, determined for the enzymic aminolysis of the above esters by H-Gly-Leu-OH, and those calculated for K_{max} are also included in this Table.

TABLE

Kinetic Parameters of the Hydrolysis and Aminolysis by H-Gly-Leu-OH of Peptide esters and the Hydrolysis of the Formed Peptide, Catalysed by Chymotrypsin and Alkaline Mesentericopeptidase, pH 9.3,25°C

Substrates	k_{cat}/K_m[a] $s^{-1}M^{-1}$	α 10^6	β	K_{max} 10^{-6}	t'_{max} 10^6 sM Exp	Calc
α-Chymotrypsin						
Ac-$Phe(NO_2)$-OMe	16 500[b]	4.5	1 450	319	750	815
Ac-$Phe(NO_2)$-Gly-Leu-OH	0.075					
Ac-Gly-$Phe(NO_2)$-OMe	106 000[e]	3.0	1 150[b]	381	150 165[c]	130
Ac-Gly-$Phe(NO_2)$-Gly-Leu-OH	0.320					
Ac-Gly-Gly-$Phe(NO_2)$-OMe	355 000[e]	2.1	1 020[b]	483	37	39
Ac-Gly-Gly-$Phe(NO_2)$-Gly-Leu-OH	0.750					
Alkaline Mesentericopeptidase						
Ac-$Phe(NO_2)$-OMe	12 100	21 500	480	0.022	300	360
Ac-$Phe(NO_2)$-Gly-Leu-OH	260					
Ac-Gly-$Phe(NO_2)$-OMe	42 100	19 700	540	0.027	90 110[d]	107
Ac-Gly-$Phe(NO_2)$-Gly-Leu-OH	830					
Ac-Gly-Gly-$Phe(NO_2)$-OMe	560 000	17 000	510	0.030	9	8
Ac-Gly-Gly-$Phe(NO_2)$-Gly-Leu-OH	9 550					

[a] Ratio of k_{cat} and K_m
[b] Taken from ref.(14)
[c] [E] = 0.5 μM; the rest of the data are obtained using 0.25 μM enzyme
[d] [E] = 0.05 μM; the rest of the data are obtained using 0.025 μM enzyme
[e] Lower values have been obtained titrimetrically (ref.14)

Due to the P_i-specificity (i = 2,3....n, Schechter and Berger notation[18]), the specificity constants k_{cat}/K_m for both peptide esters and oligopeptides increase sharply with the involvement of the P_2 and P_3 residues (Table). Their ratios, however, remain constant (mensentericopeptidase), or decrease slightly (chymotrypsin), when an extended acyl component is added. A similar effect of the secondary enzyme specificity is observed with β as well.

The β-values for the microbial enzyme and chymotrypsin are of the same order of magnitude. In contrast, the α-values differ strongly (Table). The more than three orders of magnitude difference in α gives rise to the same difference in K_{max}. Characteristically, K_{max} is practically independent of the secondary enzyme-substrate interactions for the particular proteinase. This suggests that K_{max}-

values, determined from enzymic synthesis of model peptides could be used to characterize the kinetically controlled enzymic fragment condensation.

From a preparative point of view, the time t_{max}, taken to attain the kinetically controlled maximum concentration of the peptide formed, $[RCONHR"]_{max}$, (Fig.), is one of the most important parameters in the kinetically controlled enzymic peptide synthesis. A known t_{max} allows the reaction to be stopped on time and thus the full potential of the approach can be used. Since with the present systems $[RCOOR']_o << [NH_2R"]_o$ and $[RCONHR"] << K_p$, the expression of Gololobov et al.[15b] for this parameter is valid:

$$t_{max} = \frac{(1 + \beta R_n)}{[E_o](k_{+2}/K_s)(1 + \beta R_n - \alpha)} \ln \frac{1 + \beta R_n}{\alpha} \qquad 7.$$

where R_n is the nucleophile concentration ratio $[NH_2R"]/[H_2O]$. Since $\alpha << 1$ (Table), using 3., the latter equation can be simplified and rearranged into

$$t'_{max} = \frac{\ln(\alpha^{-1} + K_{max}R_n)}{k_2/K_s} \qquad 8.$$

where $t'_{max} = t_{max}[E]_o$ is the time, taken to attain the maximum concentration using 1 mole proteinase. The maximum time characterises the effectiveness of the particular proteinase in a kinetically controlled enzymic peptide synthesis. Thus alkaline mesentericopeptidase is a better catalyst than α-chymotrypsin in the aminolysis reactions studied (Table). t'_{max} strongly depends on the proteinase secondary specificity. Furthermore, a good coincidence is observed between the maximum times obtained experimentally (Table) and those calculated using eq.8 and data for K_{max}, α and k_{+2}/K_s from the Table.

In conclusion, the relationships between the yield-determining parameters of the kinetically controlled enzymic peptide synthesis and the kinetic data for enzymic aminolysis/hydrolysis reactions enable peptide chemists to assess the best conditions for optimizing the synthetic reaction.

EXPERIMENTAL

Enzymes. Crystalline alkaline mesentericopeptidase was isolated from culture broth of Bacillus mesentericus[19]. Bovine pancreatic chymotrypsin was obtained from Boehringer Mannheim. The normality of the enzyme stock solutions was determined by the active site titration with N-transcinnamoylimidasole[20].

Substrates. Acetyl-$(Gly)_n$-Phe(NO_2)-OMe(n = 0,1,2) and acetyl-Phe(NO_2)-Gly-Leu-OH were prepared as described previously[14]. Tetra- and pentapeptide acids were synthesized by kinetically controlled chymotryptic peptide synthesis. 2 mmoles of H-Gly-Leu-OH[21] were dissolved in 5 ml 0.2 M sodium carbonate/bicarbonate buffer and pH adjusted to 9.3 with 5 N NaOH. 10 mg α-chymotrypsin was then added, followed by 1 mmole of powdered peptide ester. After the synthesis was over (disappearance of the solid phase), the resulted peptide precipitated after acidification to pH 2. Recrystallization from ethyl acetate/petroleum ether produced the pure product. Acetyl-Gly-Phe(NO_2)-Gly-Leu-OH: yield 70%; m.p. 162-165°C; $[\alpha]_D^{20}$ = 25.6(c 0.2, EtOH); Elemental analysis: found C 52.12, H 6.41, N 14.80; calcd. for $C_{21}H_{29}N_5O_8$: C 52.61, H 6.05, N 14.63; ^{1}H NMR(250 MHz, Me_2SO-d_6) data: δ 4.5 (1H, Phe(NO_2) $C^{\alpha}H$), 4.23(1H, Leu $C^{\alpha}H$), 3.74 and 3.60(4H, 2 Gly $^2C^{\alpha}H_2$), 1.83(3H, acetyl CH_3), 0.85(6H, Leu $C^{\delta}H$).
Acetyl-Gly-Gly-Phe(NO_2)-Gly-Leu-OH: Yield 65%; m.p. 170-172°C; $[\alpha]_D^{20}$ = 17.3 (c 0.2, EtOH). Elemental analysis: found C 51.12, H 6.19, N 15.30; calcd. for $C_{23}H_{32}N_6O_9$: C 51.49, H 5.97, N 15.67; ^{1}H NMR(250 MHz, Me_2SO-d_6) data: δ 4.59 (1H, Phe(NO_2) $C^{\alpha}H$), 4.23(1H, Leu $C^{\alpha}H$), 3.76, 3.66 and 3.61(6H, 3 Gly $C^{\alpha}H_2$), 1.83 (3H, acetyl CH_3), 0.85(6H, Leu $C^{\delta}H$).

Spectrophotometric analysis. The enzymic hydrolysis of the Phe(NO_2)-containing peptides and peptide esters was followed spectrophotometrically at 335 nm with a Shimadzu uv-3000 spectrophotometer. Values of the kinetic parameters k_{cat} and K_m were determined from the double-reciprocal plots of the initial rate data. Aminolysis/hydrolysis ratio of H-Gly-Leu-OH for different peptide esters were determined by the method used earlier[14]. Caution has been exercised in the case of di- and tripeptide esters to avoid ester hydrolysis during the mixing of the reagents.

HPLC analysis. Time-dependence of the peptide bond formation was studied by high-performance liquid chromatography of the samples withdrawn at different times

from the reaction mixture on a RP-18 Bondapack analytical column, WATERS delivery system 6 000 A. The elution system for the separation of the peptide ester, peptide acid and peptide was MeOH/30 mM KH_2PO_4, pH 4.6 (1:1, v/v), flow rate 1.3 ml/min. The reaction mixture contained 2 mM peptide ester, 82.5 mM H-Gly-Leu-OH, 0.25- 2.5 μM α-chymotrypsin or 0.005-0.5 μM alkaline mesentericopeptidase and 0.2 M sodium carbonate/bicarbonate buffer pH 9.3 as starting materials.

ACKNOWLEDGEMENTS

We thank Dr.V.K.Svedas (Moscow State University, Moscow, USSR), for preprints of his recent papers and to Dr.V.Kasche (Technische Universität Hamburg-Harburg, FRG) for stimulating discussions.

REFERENCES

1. M.M.Botvinik, V.I.Ostoslavskaya, L.I.Ivanov and G.K.Gorshenina, Zh.Obshchei Khim., 31, 3234(1961).
2. K.Morihara and T.Oka, Biochem.J., 163, 531(1977); b) K.Morihara, Trends Biotechnol., 5, 164(1987).
3. D.D.Petkov, J.Theor.Biol., 98, 419(1982).
4. J.S.Fruton, Adv.Enzymol. 53, 239(1982).
5. H.-D.Jakubke, The Peptides, Academic Press Inc, New York, 8, 103(1987).
6. J.Blair West and C.-H.Wong, J.Chem.Soc.,Chem.Commun. 417(1986); b) J.Blair West and C.-H.Wong, J.Org.Chem. 51, 2728(1986).
7. S.A.Bizzozero, H.Dutler, R.Franztack and B.A.Rovagnati, Helv.Chim.Acta, 68, 981(1985).
8. W.Kullmann, Enzymatic Peptide Synthesis, CRC Press, Boca Raton (1987).
9. A.L.Margolin, D.-F. Tai and A.M.Klibanov, J.Amer.Chem.Soc. 109, 7885(1987).
10. T.L.Voyushina, L.A.Lyublinskaya and V.M.Stepanov, Bioorg.Khim. 11, 738(1985).
11. F.Widmer, Protides Biol.Fluids, Proc.Colloq. 34, 27(1986).
12. Y.V.Mitin, S.A.Ogii and V.A.Tertych, Bioorg.Khim. 11, 1476(1985).
13. J.Fastrez and A.R.Fersht, Biochemistry, 12, 2025(1973).
14. D.D.Petkov and I.B.Stoineva, Biochem.Biophys.Res.Commun. 118, 317(1984); b) V.Schellenberger and H.-D.Jakubke, Biochem.Biophys.Acta, 869, 54(1986).
15. V.Kasche, Enzyme & Microb.Technol. 8, 164(1986); b) M.Y.Gololobov, I.L.Borisov and V.K.Svedas, Biokhimia (Moscow), 52, 584(1987).
16. I.Svedsen, N.Genov and K.Idakieva, FEBS Lett. 196, 228(1987).
17. M.L.Bender, G.E.Clement, C.R.Gunter and F.J.Kezdy, J.Amer.Chem.Soc. 86, 3697(1964).
18. I.Schechter and A.Berger, Biochem.Biophys.Res.Commun. 27, 157(1964).
19. M.Karadjova, A.Bakurdjieva and P.Velcheva, C.R.Acad.Bulg.Sci. 23, 431(1970).
20. C.R.Schonbaum, B.Zerner and M.L.Bender, J.Biol.Chem. 236, 515(1961).
21. K.R.Rao, S.M.Birnbaum, R.B.Kingsley and J.P.Greenstein, J.Biol.Chem. 198, 507(1952).