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Solid-phase Acyl Donor as a Substrate Pool in Kinetically Controlled Protease-Catalysed Peptide Synthesis

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Abstract: Recently we have demonstrated the advantage of solid-phase substrate pools mainly in equilibrium controlled protease-catalysed peptide syntheses. The extension of this approach to protease-catalysed acyl transfer reactions will be presented. The model reaction was systematically investigated according to both the influence of solid phases present in the system on enzyme activity as well as nucleophile concentration on peptide yield. The key parameter for obtaining high peptide yield via acyl transfer is the ratio between aminolysis and hydrolysis. We combined high nucleophile concentrations with solid-phase acyl donor pools. This approach enabled us to supply ester substrate and nucleophile in equimolar amounts in a high-density media without the addition of any organic solvent. Several multi-functional di- to tetrapeptides were obtained in moderate to high yields. ©1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Protease catalysis applied to synthetic goals has attracted remarkable attention during recent years [1–3]. The key parameter for obtaining high peptide yield via acyl transfer is the aminolysis/hydrolysis ratio. In general, in these reactions high nucleophile concentrations promote aminolysis over hydrolysis. However the solubility of the acyl donor in aqueous media is often limited [4]. Therefore, enzymic peptide syntheses are mainly performed in the presence of organic solvents to enhance educt solubility or using high nucleophile excess [5–7]. The use of organic solvents very often leads to a diminution of protein structure and enzyme activity [8–10]. Despite efforts in stabilizing enzymes [11, 12] some disadvantages are obvious: organic cosolvents cause a drastic reduction of catalytic velocities and to a large extent a loss in specificity and selectivity. Additionally, the need for disposal of organic solvents used is a disadvantage in the sense of economy and ecology. The application of solid-phase substrate pools would allow an equimolar supply of substrates combined with easy work-up procedures and compatibility with standard chemical strategies. We recently demonstrated the advantages of using solid-phase substrate pools mainly in equilibrium controlled protease-catalysed peptide syntheses [13, 14]. Now we report on the extension of this approach on acyl transfer reactions catalysed by serine and cysteine proteases. The model system was systematically investigated according to the influence of solid phases present in the reaction system on enzyme activity as well as the nucleophile concentration on peptide yield. The studies were performed with varying sequences of a tripeptide with potential growth factor activities H-Xaa-His-Xbb-OH

Abbreviations: DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic) acid; -OCam, carboxamidomethyl ester; -ONb, 4-nitrobenzyl ester; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TLCK, tosyl-lysyl-chlormethylketone; Xaa, Xbb, amino acids; Y′, Y″ amino- and carboxy protection groups, respectively.

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(Xaa = Ala, Arg, Gly, Lys, Ser; Xbb = Arg, His, Lys) and a tetrapeptide sequence found in ACTH and α-MSH (-His-Phe-Arg-Trp-), respectively.

MATERIALS AND METHODS

Materials

Z-Xaa-OCam (Xaa = Ala, Gly, Ser) was synthesized from Z-Xaa-OH and iodacetamide as described by Martinez *et al.* [15]. Esterification of H-His-OH*2 $BzSO_3H$ with HONb to obtain H-His-ONb*2 $BzSO_3H$ was performed according to Shields *et al.* [16]. All other amino acids derivatives were purchased from BACHEM Biochemica GmbH (Heidelberg, Germany) or taken from our laboratory stock. Iodacetamide, benzol sulphonic acid, EDTA and 4-nitrobenzylalcohol were delivered by Fluka Chemie (Neu-Ulm, Germany). HEPES, TAPS and DTE were obtained from Serva Feinbiochemica GmbH & Co KG (Heidelberg, Germany). Proteases were delivered by the following suppliers: α-chymotrypsin and Papain by Merck KGaA (Darmstadt, Germany), TLCK-treated α-chymotrypsin by Fluka Chemie (Neu-Ulm, Germany) and trypsin by Serva Feinbiochemica GmbH & Co KG (Heidelberg, Germany).

Hydrolysis Kinetics in Solution and Suspension

The saturation concentration of Ac-Tyr-OEt in 2 M aqueous KCl was determined by means of HPLC (Shimadzu LC-10A) using external standards. The column, Lichrospher RP 18 (5 μm, 250 × 4 mm) purchased from Merck, was eluted isocratically at a flow rate of 0.8 ml/min. The eluent contained 15% (v/v) aqueous acetonitrile and 0.1% (v/v) trifluoroacetic acid. Detection wavelength was set to 280 nm.

Determination of *p*-Values

To 1 ml of a 5.8 mM Ac-Tyr-OEt in 2 M aqueous KCl-solution, 0.2 ml buffer (0.1 M HEPES/Na^+, pH 7.9, 2 M KCl) and 0.7 ml nucleophile solution were added. The nucleophile stock solution contained 414 mM H-Arg-NH_2*2HCl and 1.035 M H-Gly-Gly-OH in 2 M aqueous KCl, respectively. The pH of the nucleophile solution was adjusted to 7.9 with 10 M KOH. The reaction was started by addition of 0.1 ml enzyme solution (2 mg α-chymotrypsin/ml in 2 M aqueous KCl). To follow the reaction progress aliquots were withdrawn, diluted with 1 ml 50% (v/v) aqueous acetonitrile containing 2% (v/v) acetic acid and analysed with the HPLC system as described. The column was eluted by a gradient as follows: 0–2 min eluent A, 2–25 min linear gradient to 100% B. A contained 5% (v/v) acetonitrile, 0.1 (v/v) trifluoroacetic acid and water. B consisted of 25% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and water. The flow rate was kept constant at 0.8 ml/min and the detection wavelength was 280 nm. The composition of reaction mixture after appropriate time intervals was used to calculate the *p* values in the procedure described by Schellenberger and Jakubke [17]. *p*-Values of papain-catalysed acyl transfer reactions of Z-Gly-OCam towards H-His-Y (Y = NH_2, OMe, ONb) were determined similarly.

Synthesis of Model Peptides

In a typical experiment 25.1 mg (100 μmol) Ac-Tyr-OEt were suspended in 100 μl of 1 M H-Arg-NH_2*2HCl (pH adjusted to 9.15 with 10 M KOH) stock solution in buffer. Some 80 μl of buffer containing 1.25% (v/v) Triton X-100 were added. The reaction was started by addition of 20 μl of 0.25 mg/ml α-chymotrypsin solution in buffer. The reaction was followed by HPLC as described above.

Synthesis of Z-His-Phe-Arg-Trp-NH_2

Z-Arg-Trp-NH_2 was obtained in a trypsin-catalysed reaction similar to the procedure described above (for amounts see Table 1). Z-Arg-Trp-NH_2 was purified by preparative HPLC. The HPLC was equipped with a column Vydac 201HS1022 (10 μm, 90 Å, 22 × 250 mm). The column was isocratically eluted with 30% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 15 ml/min. Z-Arg-Trp-NH_2 fractions were pooled and freeze dried.

Some 15 mg of Pd/C-catalyst were suspended in 25 ml methanol and activated by subsequent bubbling with N_2, H_2 and N_2 for 10 min each. 364 mg (0.6 mmol) Z-Arg-Trp-NH_2*TFA salt diluted in 5 ml of methanol was added to catalyst suspension and flushed with N_2 for 10 min followed by H_2 for 5 h. After that the catalyst was removed by filtration and the solution was evaporated under reduced pressure. The residue was dissolved in 1.5 ml of 1 M aqueous HCl and freeze dried. Z-His-Phe-Arg-Trp-NH_2 was synthesized similarly as described above (for amounts see Table 1). The product, Z-His-Phe-Arg-Trp-NH_2, was purified by preparative HPLC as described.

Table 1 Yields of α-Chymotrypsin and Papain-catalysed Peptide Syntheses in High-density Media

Substrate	Nucleophile	S/V[a] (mol/l)	Time (h)	Yield (%)
Ac-Tyr-OEt[b]	H-Arg-NH_2	0.5	1	90
		1	1	90
	H-Gly-Gly-OH	0.5	1	58
		1	2	63
	H-Ala-Ile-OH	1	3	86
Z-His-Phe-OBzl[c]	H-Arg-NH_2	0.5	2	77
	H-Leu-NH_2			73
	H-Arg-Trp-NH_2	0.55	2.5	95
Z-Arg-OMe[d]	H-Trp-NH_2	1.67	2	47
Z-Gly-OCam[e]	H-His-OMe	0.5	0.25	90
	H-His-ONb[e]		0.3	84
Z-Ser-OCam[f]	H-His-ONb	0.5	1	86
Z-Ala-OCam[f]*			5	77
Z-Arg-OMe[f]			5	74
Z-Lys-OMe[f]*			1	75
Z-Gly-His-ONb[g]	H-Arg-NH_2	1	6	75
	H-His-NH_2		6	50
	H-Lys-NH_2		1.5	90
Z-Ser-His-ONb[g]*	H-Arg-NH_2	1	8	85
	H-Lys-NH_2			76

[a]S/V: ratio of substrates to volume of aqueous phase used. Reaction conditions: [b]100 μmol of each, substrate and nucleophile, [NH_4CH_3COO-buffer, pH 9.15] = 0.5 M, 0.5% (v/v) triton X-100; 0.22 nmol α-chymotrypsin, [c]50 μmol of each, substrate and nucleophile, [HEPES/Na^+-buffer, pH 8] = 0.5 M, 0.5% (v/v) triton X-100; 0.25 nmol α-chymotrypsin; [d]2,5 mmol Z-Arg-OMe*HCl, H-Trp-NH_2*HCl, [HEPES/Na^+-buffer, pH 8] = 0.5 M; 10 nmol trypsin; [e]0.2 mmol of each, substrate and H-His-OMe*HCl, [HEPES/Na^+-buffer, pH 7.9] = 1 M; 32 nmol papain, 2 μl triton X-100, [EDTA] = 10 μM, [DTE] = 0.25 mM; [f]0.1 mmol of both, substrate and H-His-ONb*$BzSO_3H$, [HEPES/Na^+-buffer, pH 7.9] = 1 M; 0.2 μmol (*0.02 μmol) papain, 2 μl triton X-100, [EDTA] = 20 μM, [DTE] = 0.5 mM; [g]0.1 mmol acyldonor and nucleophile dihydrochloride salt, [TAPS/Na^+-buffer, pH 9] = 0.5 M; 15 nmol α-chymotrypsin (*30 nmol TLCK-α-chymotrypsin), 2 μl triton X-100.

Synthesis of Z-Xaa-His-Xbb-NH_2

The indicated amount (see Table 1) of Z-Xaa-OCam (Xaa = Ala, Gly, Ser) was suspended in an appropriate amount of buffer, containing an equimolar amount of H-His-OR (R = Me, Nb) salt. Concentrated aqueous ammonium hydroxide solution was used to release the nucleophile from its salt and to adjust the pH. After addition of the indicated amount of Triton X-100 the reaction was started by addition of Papain solution in buffer containing the appropriate amount of EDTA and DTE. The reaction mixture was intensively mixed. Samples were taken after several time intervals and analysed by HPLC on the Shimadzu LC 10 A system to follow the reaction's progress. After complete ester consumption the reaction mixture was dissolved in methanol and heated to inactivate the enzyme. After filtration the product Z-Xaa-His-ONb was precipitated by addition of about three volumes of distilled water. After completing the precipitation at 4°C overnight the peptide product was filtered off and dried over P_4O_{10} under reduced pressure. In the case of Z-Xaa-OMe (Xaa = Arg, Lys) the peptide was purified by preparative HPLC as described above.

In analogy to the procedure described, the α-chymotrypsin-catalysed chain prolongation was performed. The final tripeptide derivative was obtained as a trifluoroacetate salt after preparative HPLC purification and evaporation of the solvent.

RESULTS AND DISCUSSION

As a model system the α-chymotrypsin-catalysed synthesis of Ac-Tyr-X (X = Arg-NH_2, Gly-Gly-OH) from Ac-Tyr-OEt and H-X was chosen. Two nucleophiles were selected as examples of an efficient (H-Arg-NH_2) and a poor nucleophile (H-Gly-Gly-OH), respectively.

The α-chymotrypsin-catalysed hydrolysis of Ac-Tyr-OEt both in solution and with solid ester particles present was first investigated. Surprisingly, the specific reaction rate decreased with increasing amount of solid particles present. Furthermore, the reproducibility decreased from ±5% in solution to ±50% in heterogeneous media. The use of ester particles of smaller size do not lead to hydrolysis rates as high as in solution or to higher

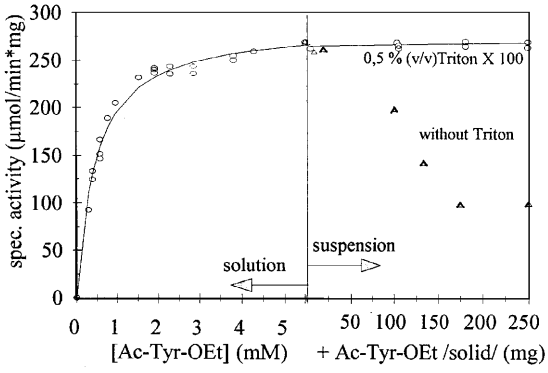

Figure 1 Kinetics of α-chymotrypsin-catalysed hydrolysis of Ac-Tyr-OEt in solution (left side) and suspension (right side). The indicated amount (mg) of solid Ac-Tyr-OEt was added to a saturated solution. Reaction conditions: [α-chymotrypsin] = 10 nM, [KCl] = 2 M, ϑ = 25°C, pH 7.9, titration with [NaOH] = 20 mM.

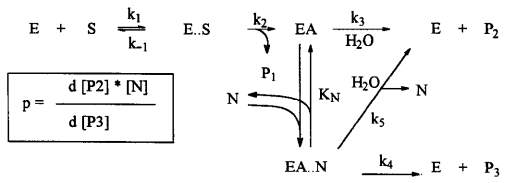

Figure 2 Kinetic scheme of protease-catalysed acyl transfer reactions; E, free enzyme; S, ester substrate (Y′-Xaa-OR); E..S, enzyme-substrate complex; P_1, leaving group (HOR); P_2, hydrolysis product (Y′-Xaa-OH); P_3, aminolysis product (Y′-Xaa-Xbb-Y″); N, nucleophile (H-Xbb-Y″); EA, acylenzyme; EA..N, acylenzyme–nucleophile complex.

reproducibility. From this observation we may conclude that mass transfer from the solid ester to solution is not a limiting factor. The addition of a detergent to guarantee complete wetting of ester particles increased reaction velocity and reproducibility to values found in a saturated solution as shown in Figure 1.

For H-Arg-NH_2 and H-Gly-Gly-OH the nucleophile efficiency was determined via acyl transfer using Ac-Tyr-OEt as acyl donor. *p*-Values were introduced by Könnecke *et al.* [18] in similarity to the Michaelis constant and can serve as a tool to predict the success of a kinetically controlled peptide bond formation [18, 19]. The reaction mechanism must be sufficiently described by the scheme in Figure 2. *p* values compiled in Table 2 were obtained according to Schellenberger and Jakubke [17] by means of weighted linear regression analysis. The papain-catalysed acyl transfer of Z-Gly-OCam to H-His-Y (Y = NH_2, OMe, ONb) was included here as a second model. p_N (k_5/k_4) of about zero for nucleophiles in both systems reveals that the hydrolysis of the acyl enzyme nucleophile complex does not play a significant role. Therefore, if the enzyme can be saturated with nucleophile, high yields can be obtained.

Table 2 Effective *p*-Values of α-Chymotrypsin-catalysed Acyl Transfer of Ac-Tyr-OEt to H-Arg-NH_2 and H-Gly-Gly-OH and of Papain-catalysed Acyl Transfer of Z-Gly-OCam to H-His-Y (Y = NH_2, OMe, ONb)

Nucleophile	$p_0 = \frac{K_N k_3}{k_4}$ (mM)	$p_N = \frac{k_5}{k_4}$
H-Arg-NH_2[a]	0.512 ± 0.039	0.0296 ± 0.0017
H-Gly-Gly-OH[a]	96.7 ± 1.4	0.0045 ± 0.0163
H-His-NH_2[b]	5.38 ± 0.33	0.072 ± 0.352
H-His-OMe[b]	2.78 ± 0.12	0.080 ± 0.337
H-His-ONb[b]	0.03 ± 0.05	0.040 ± 0.080

Reaction conditions: [a][Ac-Tyr-OEt] = 2.9 mM, [α-chymotrypsin] = 4 nM, [KCl] = 2 M, ϑ = 25°C, pH 7.9 [Hepes/Na^+] = 20 mM, [H-Arg-NH_2] = 2.9...290 mM, [H-Gly-Gly-OH] = 14.5...725 mM; [b][Z-Gly-OCam] = 0.5 mM, [Papain] = 0.8 μM, [KCl] = 2 M, [H-His-Y (Y = NH_2, OMe, ONb)] = 0.5...25 mM, pH 7.9 [Hepes/Na^+] = 100 mM, = 25°C, [EDTA] = 20 μM, [DTE] = 0.5 mM.

Finally, we combined high nucleophile concentrations with solid-phase-ester pools. This approach enabled us to supply the ester substrate and nucleophile equimolar in a high-density media. Yields obtained by this approach using solid-phase substrate pools are compiled in Table 1. As expected from a low p_o value, Ac-Tyr-Arg-NH_2 was obtained in a high yield even at pH 7.9. At this pH Ac-Tyr-Gly-Gly-OH could be attained only in 34% yield. The reason was a drop in pH causing a decrease in selectivity (ratio between desired peptide product and ester substrate consumed at a certain time) from 60 to 34%, starting at 20% ester consumption. Initial pH was raised to 9.15 using an ammonium acetate buffer. Ac-Tyr-OEt was then transferred to the very poor nucleophile H-Gly-Gly-OH in acceptable yield. During reaction the pH decreased to 8.5 at complete ester conversion. Selectivity varied between 63 and 67%.

This approach using solid-phase substrate pools was applied to the synthesis of di- to tetrapeptide derivatives. The tetrapeptide Z-His-Phe-Arg-Trp-NH_2 was synthesized as follows. Z-His-Phe-OBzl was obtained in 84% yield via thermolysin catalysis, as described recently [14]. Z-Arg-OEt was coupled with H-Trp-NH_2 in 47% yield in a trypsin-catalysed reaction. After hydrogenolytic deprotection of Z-Arg-Trp-NH_2, Z-His-Phe-OBzl and H-Arg-Trp-NH_2 were assembled to give Z-His-Phe-Arg-Trp-NH_2 by means of α-chymotrypsin in 95% yield.

Furthermore, Z-Xaa-His-Xbb-NH_2 (Xaa = Ala, Arg, Gly, Lys, Ser; Xbb = Arg, His, Lys), precursors of peptides of the common structure H-Xaa-His-Xbb-OH having broad biological activity, was obtained in two enzymatic steps. Some of these tripeptides are reported to influence growth of several differentiated cells [20]. For example liver cells are stimulated by the growth factor H-Gly-His-Lys-OH [21] whilst bursin (H-Lys-His-Gly-NH_2) is a selective B-cell-differentiating hormone isolated from the bursa of Fabricius [22].

The papain-catalysed synthesis of Z-Xaa-His-ONb was directly followed by α-chymotrypsin-catalysed chain prolongation to yield the final tripeptide derivative. Z-Xaa-OCam and Z-Xaa-OMe, respectively, served as acyl donors in the first step. According to the results of HPLC Z-Gly-His-ONb

was obtained in 84% yield and isolated in 76% yield, respectively. The nucleophile H-His-ONb was advantageous over H-His-OMe or H-His-NH_2 for three reasons. Firstly, the 4-nitrobenzyl group contributed to binding of the nucleophile to the active site of papain in P_2'-position as can be concluded from very low p_0 values compared with methyl or amide group. Secondly, the hydrophobic product crystallized readily from the aqueous reaction media. Thirdly, the nitrobenzyl moiety is a good leaving group in subsequent chain prolongation by, for example, α-chymotrypsin [23]. The dipeptide ester obtained was directly applied to α-chymotrypsin-catalysed transfer reaction to H-Arg-NH_2, H-His-NH_2 and H-Lys-NH_2, respectively. In the case of Z-Ser-His-ONb as ester substrate, a TLCK-treated chymotrypsin preparation was used to avoid hydrolytic side reactions (deamidation of product Z-Ser-His-Arg-NH_2) by traces of trypsin in chymotrypsin. Further analytical results of these coupling reactions are noted in Table 1 among other enzymic peptide syntheses.

In all cases organic solvents could be completely avoided. The results show that a limited reactant solubility does not prevent good yields or complete conversion. Our findings agree with literature results [24, 25].

CONCLUSIONS

The key parameter for obtaining a high peptide yield in kinetically controlled protease-catalysed peptide synthesis is the aminolysis/hydrolysis ratio. High nucleophile concentrations promote aminolysis over hydrolysis. The application of solid-phase substrate pools combines the equimolar supply of substrates with high product yields, easy work-up procedures and compatibility with standard chemical procedures. The suitability of this approach was demonstrated with the protease-catalysed synthesis of two potential biologically active peptide derivatives. The multifunctional peptides Z-His-Phe-Arg-Trp-NH_2 and Z-Xaa-His-Xbb-NH_2 (Xaa = Ala, Arg, Gly, Lys, Ser; Xbb = Arg, His, Lys) with subsequent trifunctional amino acid residues were even successfully synthesized as model peptides without any side-chain protection. Organic solvents could be completely avoided.

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