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Synthesis of Dipeptides by Suspension-to-suspension Conversion via Thermolysin Catalysis: from Analytical to Preparative Scale

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Abstract: When using proteases in direct reversal of their normal hydrolytic function, the equilibrium position is very important in limiting the attainable yield in equilibrium-controlled enzymic peptide synthesis. Analysis of the equilibrium position reveals a favourable shift towards the peptide product if starting materials are largely undissolved in the reaction medium and the product precipitates. This approach enabled us to obtain high peptide yields in thermolysin-catalysed reactions in high-density aqueous media with an equimolar supply of substrates. The easy scale-up (up to mol-scale) of this approach is demonstrated by two examples. Z-His-Phe-NH_2 and Z-Asp-Phe-OMe, precursors for cyclo-[-His-Phe-] and the low-calorie sweetener Aspartame, respectively, were synthesized in preparative yields of 84–88%. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Enzymic methods of peptide bond formation broaden the methodological repertoire of peptide chemists. In particular, mild reaction conditions guaranteeing both racemization-free synthesis and environmentally acceptable reaction systems are advantages of enzymic methods. Kinetically controlled transfer reactions are limited to serine and cysteine proteases with slightly activated acyl donor esters as prerequisites. However, when proteases are used in direct reversal of their normal hydrolytic function, the equilibrium position is very important in limiting the attainable yield in thermodynamically controlled enzymic peptide synthesis. The increased solubility of amino acid derivatives sparingly soluble in purely aqueous media, shifting the equilibrium position towards the desired peptide product, and the minimization of hydrolytic side reactions are the main reasons for the addition of organic cosolvent. These reasons extend to homogeneous and heterogenous, aqueous organic media and low water organic reaction systems, respectively [2–14]. Substitution of water by organic solvents often leads to destabilization of protein structure and loss of enzyme activity [15–17].

The analysis of equilibrium position revealed a favourable shift towards the peptide products, when starting materials are largely undissolved in the reaction medium and the product precipitates [18]. The consideration resulted in water-based high-density media. This approach enabled us to obtain high peptide yields in high-density aqueous media with equimolar supply of substrates. The reaction system was scaled up from analytical to multi-mol pilot scale.

Abbreviations: ACO-, Aminocarbonyl-; Npg, Neopentylglycine; Nvl, Norvaline; Phg, Phenylglycine; Tle, *tert*-Leucine[1]; Y,Y′, protection groups; Xaa, Xbb, amino acids.

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MATERIAL AND METHODS

Analytical Scale Experiments

In a typical experiment 0.1 mmol of both starting materials were placed in a polyethylene tube and intensively mixed with 20 μl of buffer at 40°C. NaOH was added if necessary to release free nucleophile base from its salt until a pH as indicated was obtained. The reaction was started with the indicated amount of thermolysin suspended in 10 μl buffer. Samples of reaction mixture were dissolved in a mixture of 0.5 ml acetonitrile and 0.5 ml 5% aqueous trifluoroacetic acid, filtered and analysed by a Shimadzu LC-6A HPLC system supplied with an integrator CR-4AX. A Merck-Lichrochart RP 18 column (250 × 4 mm^2) was eluted isocratically. The eluent contained varying amounts of acetonitrile. The aqueous phase of the eluent contained 0.1% trifluoroacetic acid. The peptide products were identified by comparison with authentic or characterized samples. For isolation of peptide products the reactions were scaled up to 1 mmol. The precipitated product was washed with water and recrystallized from methanol/water. Finally, the product was dried over P_4O_{10} in a vacuum desiccator.

Synthesis of cyclo-(-His-Phe-)

Z-His-OH and H-Phe-NH_2*HCl were placed in a pilot-scale mixer M5R (Gebr. Lödige Maschinenbau GmbH, Bremen, Germany) with a volume of 5l. Amounts are indicated in Table 2. Buffer and 10 M NaOH were added and the resulting suspension was stirred at 200 r.p.m. After heating to 40°C, the mixer speed was lowered to 100 r.p.m. and the reaction was started by addition of enzyme. Samples were taken, dissolved in 0.3 ml acetonitrile and 0.7 ml 5% (v/v) aqueous trifluoroacetic acid, and analysed by HPLC. After a reaction time of 6.5 h, the precipitate was washed with 10 l ice-cold water and dried under vacuum at 55°C.

Some 0.2 mol Z-His-Phe-NH_2 were deprotected with H_2 over Pd/C in 1.5 l methanol in the presence of 0.44 mol acetic acid. After careful filtration, the solvent was evaporated under reduced pressure. The crystallized H-His-Phe-NH_2*$(CH_3COOH)_2$ was dried under vacuum in a desiccator over P_4O_{10}.

Some 50 mmol H-His-Phe-NH_2*$(CH_3COOH)_2$ were dissolved in 250 ml methanol, 2 equivalents *N*-ethylmorpholine added and refluxed. The precipitated cyclo-[-His-Phe-] was washed several times with methanol and dried in a desiccator under reduced pressure.

Synthesis of Z-Aspartame

Z-Asp-OH was transferred to the pilot-scale mixer M5R (Gebr. Lödige Maschinenbau GmbH, Bremen, Germany) and mixed with buffer or water as indicated. The NaOH was added as micro pills and stirred intensively. While the reactor was cooled with ice the Z-Asp-disodium salt precipitated. H-Phe-OMe*HCl was added, mixed and the reactor was heated to 40°C. The reaction was started by the addition of thermolysin. Samples were taken as described above and analysed by HPLC. After 7 h, the precipitate was washed with water and either dried in a vacuum oven at 55°C (exp. 5) or the wet cake was suspended in ~10 l water (exp. 4). Then, in exp. 4, the pH was adjusted with concentrated HCl to pH 2 and stirred for 6 h. If necessary, the pH was readjusted. The slurry was filtered and washed with water until the filtrate showed a neutral reaction. The filtrate was dried as above. Z-group removal was performed according to Degussa procedures.

Materials

ACO-Tyr-OH, ACO-Met-OH, ACO-Leu-OH, H-Phe-NH_2*HCl, H-Phe-OMe*HCl, H-Tle-NH_2, Z-Asp-OH and Z-His-OH were products of Rexim (Courbevoie, France). H-Npg-NH_2*HCl was donated by Dr Georg Krix, IET Jülich. All other amino acid derivatives were purchased from Bachem Feinchemikalien AG (Buchs, Switzerland) or taken from our laboratory stock. Thermolysin was delivered as Protease Type X by Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

Theoretical

A complete theoretical treatment has been provided in [18]. In this section only the main ideas and arguments of the suspension-to-suspension approach to peptide synthesis are presented. The equilibrium of a reverse peptide hydrolysis reaction with the following scheme is to be analysed:

$$A + B \rightleftarrows P + H_2O$$

where A is Y-Xaa-OH; B is H-Xbb-Y′ and P is Y-Xaa-Xbb-Y′.

A general equilibrium constant K in the liquid phase can be defined as the quotient of activities (a) of starting materials and products:

$$K = \frac{a_P \cdot a_W}{a_A \cdot a_B} \quad (1)$$

In a system in which reactants other than water (w) are present as an excess of undissolved solids, additional solubility equilibria have to be considered. If dissolved and undissolved material of one reactant coexist, the activity of this species in the liquid phase is constant independent on the ratio of dissolved to undissolved reactants:

$$\begin{array}{ccccc} A_d & + & B_d & \rightleftarrows & P_d + H_2O \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ A_s & & B_s & & P_s \end{array}$$

where d is dissolved and s undissolved species.

If A, B and P are present in amounts exceeding their respective solubility limits, the activities of all three partners A, B and P become constant. In this situation, there is only one value of the water activity a_w which is consistent with the equilibrium constant K. Exactly one critical value of the water activity exists at which Eq. (1) becomes valid. Assuming the reaction is performed in aqueous media at a constant water activity equal to 1, at least one of the other activities must decrease below the value of K in a saturated solution. Two cases can be distinguished:

Case I:

$$K < \frac{a_P^\circ \cdot a_W}{a_A^\circ \cdot a_B^\circ} \quad (2)$$

The equilibrium activity of P can never reach its highest value in a saturated solution. The equilibrium position lies far on the hydrolysis side. *Nearly no conversion* to peptide product is observed.

Case II:

$$K > \frac{a_P^\circ \cdot a_W}{a_A^\circ \cdot a_B^\circ} \quad (3)$$

The activity of P attains its highest value. The reaction must continue as long as at least one of the substrates become unsaturated, so that the appropriate activity can decrease. An *almost complete conversion* may be obtained, if the reaction is started from mainly undissolved substrates.

In principle a reaction system starting with mainly undissolved substrates can reach equilibrium in two positions as described above. Either almost no product is yielded (case I) or nearly 100% product is attainable (case II). This behaviour is called *switch-like behaviour*.

A minimal liquid phase is inevitable since enzymic catalysis was shown to occur mostly in liquid phase, if not exclusively [19]. Largely undissolved substrates serve as a solid-phase pool from which consumed substrate can be replaced. Provided the peptide product precipitates at all, the conversion should continue until at least one of the substrate pools disappears completely. Using such a pool may shift the overall equilibrium far towards the peptide product even if the reaction is performed in high-density aqueous media with equimolar supply of substrates.

RESULTS AND DISCUSSION

Recently heterogeneous systems were successfully applied to enzymic peptide synthesis [20–25]. Well-established chemical protection groups allow only a poor solubility of amino acid and peptide derivatives in water, a fact that favours solid-phase substrate pool formation in aqueous media. For reasons discussed above, high peptide yields are expected if the reaction proceeds from mainly undissolved starting materials. Therefore, we performed thermolysin-catalysed coupling of several N^α-protected amino acids with amino acid amides in a suspension-to-suspension approach using a small amount of aqueous buffer. As Table 1 demonstrates, high yields were obtained without any organic solvent added. Thermolysin also catalysed the coupling of L-phenyllactic acid, a recently discovered component of sea anemones neuropeptides [26], with leucine amide to give H-L-Phlac-Leu-NH_2. The basic and acidic peptide derivatives Z-Xaa-Leu-NH_2 (Xaa = Asp, Arg, Lys) yielded only 74, 55 and 60%, respectively, because these peptides did not precipitate from the reaction mixture. Z-ΔAla-OH and Z-βAla-OH could not be coupled with H-Leu-NH_2 owing to the kinetic limitations of thermolysin. All types of amino acids such as hydrophobic, polar, basic and acidic α-amino acids are accepted as carboxy components. As nucleophiles only amino acids with hydrophobic residues could be coupled with Z-Phe-OH successfully. Thermolysin did not accept H-His-NH_2 at all, Z-Phe-Ser-NH_2 and Z-Phe-Gly-NH_2 could only be attained in yields of about 10% within five days. Owing to steric hindrance,

Z-Phe-Tle-NH_2 was obtained in traces only. Also attempts to couple nucleophiles with larger residues than benzyl (as Tyr, Trp) with Z-Phe-OH lead only to minimal formation of peptide. These residues are too big to fit in the hydrophobic binding pocket in a productive mode [27–34]. The observed behaviour of thermolysin agrees well with the specificity of thermolysin to cleave Xaa-Phe(Leu, Ile) bonds.

The positive results encouraged us to scale-up the reaction system. Cyclo-[-His-Phe-] is known as a catalyst for addition of cyanide to aldehydes with high enantiomeric excess [35, 36]. Optically active cyanohydrins are valuable starting materials for synthesis of a variety of chiral, biologically active compounds [37–40]. As precursor for cyclo-[-His-Phe-] Z-His-Phe-NH_2 was chosen for synthesis, to be obtained in a thermolysin-catalysed reaction from Z-His-OH and H-Phe-NH_2. Employing the more expensive amide substrate instead of the amino acid ester was justified because thermolysin couples a carboxy component to an amino acid amide about one order of magnitude faster than to the corresponding amino acid ester.

Table 1 Thermolysin-catalysed Synthesis of Various Protected Peptides in High-density Media

Product		Time	Yield (%)
Ac-Phe-Leu-NH_2		1.5 h	95
ACO-Leu-Phe-NH_2	a,b,c	3 d	97
ACO-Leu-Phe-OMe	a,c,d	14 h	81
ACO-Met-Phe-NH_2	a,b,c	3 h	96
ACO-Tyr-Ile-Gly-OMe	a,b,c	3 h	89
ACO-Tyr-Nvl-NH_2	a,b,c	14 h	88
ACO-Tyr-Phe-NH_2	a,b,c	1 h	86
For-Phe-Leu-NH_2		24 h	97
H-L-Phlac-Leu-NH_2	e	28 h	95
Z-Abu-Leu-NH_2		1.5 h	95
Z-Ala-Leu-NH_2		0.5 h	95
Z-Cys(Z-Cys-OH)-Leu-NH_2	e,f	2 h	90
Z-Gln-Leu-NH_2		4 h	94
Z-His-Leu-NH_2	c,g	3 h	95
Z-homoPhe-Leu-NH_2	e	3.5 h	91
Z-Phg-Leu-NH_2	e	2 h	89
Z-Pro-Leu-NH_2		5 d	91
Z-Ser-Leu-NH_2		2.5 h	89
Z-Phe-Ala-NH_2	b,c,e	24 h	91
Z-Phe-Ile-NH_2	b,c,e	2 h	95
Z-Phe-Npg-NH_2	b,c,e	17 h	56
Z-Phe-Nvl-NH_2	b,c,e	1 h	92
Z-Phe-Leu-NH_2		0.5 h	94
Z-Phe-Met-NH_2	b,c,e	24 h	88
Z-Phe-Phe-NH_2	b,c,e	1 h	95
Z-Phe-Val-NH_2	b,c,e	3 h	82

Conditions: 0.1 mmol ([a]0.2 mmol) of each carboxy component and nucleophile, [f]0.05 mmol carboxy component, 0.5 mg ([b]1 mg, [d]2 mg) thermolysin, 40°C, 30 µl([a,e]50 µl) buffer (0.5 M Na^+-HEPES, pH 7), [c]nucleophile was added as hydrochloride, then, ([a]24 µl 10 M NaOH, [g]10 µl 9.5 M NaOH) 12 µl 10 M NaOH were used to release the nucleophile from its hydrochloride salt.

For large-scale synthesis, a pilot-scale mixer was charged with a reaction volume of 1.5 l (minimal reaction volume possible). The enzyme/substrate ratio had been optimized previously at the 10 mmol scale. Results of three runs are summarized in Table 2.

The Z-group was cleaved with H_2 over Pd/C in methanol. Under alkaline cleavage conditions the deprotected dipeptide amide did not spontaneously form the dioxopiperazine even at a slightly elevated temperature (35 °C). A second portion of Z-His-Phe-NH_2 was deprotected with 2.2 equivalents of acetic acid added. H-His-Phe-NH_2 could easily be isolated as acetate salt and the dioxopiperazine was yielded in 79% from refluxing methanol. Here *N*-ethylmorpholine was used to release the peptide from its salt.

Furthermore, the established reaction system was applied to the thermolysin-catalysed coupling of Z-Asp-OH and H-Phe-OMe to yield Z-Aspartame, an artificial sweetener precursor. Since Z-Aspartame forms an adduct with H-Phe-OMe precipitating from aqueous media two equivalents of H-Phe-OMe were supplied. To attain a favourable pH in the reaction mixture Z-Asp-OH was neutralized with NaOH in advance. The sodium salt of Z-Asp-OH then served to neutralize H-Phe-OMe*HCl very mildly and hydrolysis of nucleophile ester did not exceed 5% during reaction. In both experiments performed, nearly 95% yield was obtained according to HPLC. Preparative yields are given in Table 3.

CONCLUSIONS

The proposed reaction system applied to equilibrium-controlled enzymic peptide syntheses is characterized by solid-phase substrate pools. Thermodynamic considerations have revealed a favourable equilibrium shift towards the desired peptide product if the synthesis reaction is started from mainly undissolved substrates and the product precipitates. High dipeptide yields were obtained with economical equimolar supply of substrates in a variety of thermolysin-catalysed peptide synthesis reactions on analytical scale. No indications of an

Table 2 Reaction Conditions and Preparative Yields of Thermolysin-catalysed Formation of Z-His-Phe-NH_2 from Z-His-OH and H-Phe-NH_2 in a Pilot-scale Mixer Lödige-M5R

	Exp. 1	Exp. 2	Exp. 3
Z-His-OH/buffer-ratio	1 mol/l	3 mol/l	5 mol/l
Z-His-OH	1.03 mol (229 g)	1.91 mol (553 g)	2.31 mol 668 g)
H-Phe-NH_2*HCl	1.03 mol (208 g)	1.91 mol (383 g)	2.31 mol (463 g)
10 M NaOH	98 ml	181 ml	218 ml
Tris/HCl buffer (0.5 M, pH 7 (40 °C))	937 ml	457 ml	243 ml
Thermolysin	2.07 g	3.83 g	4.62 g
Yield	398.4 g (0.914 mol 88%	718.8 g 1.65 mol 86%	795.9 (+92.6)[a] g 1.83 (+0.213) mol 88%

[a]Reaction was interrupted at ~70% conversion. Additional product (amount given in parentheses) was obtained from washing liquor.
Conditions: 1.5 l reaction volume, 2 g thermolysin per mol Z-His-OH, 40 °C, pH 6–7, pilot scale mixer Lödige M5R (100 r.p.m.).

Table 3 Reaction Conditions and Results of Thermolysin-catalysed Synthesis of Z-Aspartame Starting from Z-Asp-OH and H-Phe-OMe*HCl

	Exp. 4	Exp. 5
Z-Asp-OH/buffer ratio	5 mol/l	5 mol/l
Buffer (1 M Tris/HCl pH 7 (40°C)	333 ml	
Water	150 ml	483 ml
Product yield	Z-Aspartame 1.094 g; 2.55 mol 84%	Z-Aspartame*H-Phe-OMe[a] 1.645 g; 2.7 mol 90%

[a]Contains small amounts of inorganic salts.
Conditions: 3 mol (801 g) Z-Asp-OH, 6 mol (1.294 g) H-PHe-OMe*HCl, 6.48 mol (259 g) NaOH, 15 g thermolysin, 35–45 °C, pilot-scale mixer Lödige M5R (100 r.p.m.)

alteration in specificity were observed. The system can easily be scaled-up as demonstrated by two examples, the synthesis of Z-His-Phe-NH_2 from Z-His-OH and H-Phe-NH_2 and the coupling of Z-Asp-OH and H-Phe-OMe to the artificial sweetener precursor Z-Aspartame. The proposed method attracts further attention by simple work-up procedures and its compatibility with well established chemical protection/deprotection methods. Organic cosolvent is either completely avoidable or can be reduced to a minimum to prevent hydrolysis of preformed peptide bonds.

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