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Reaction Medium Engineering in Enzymatic Peptide Fragment Condensation: Synthesis of Eledoisin and LH-RH

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Abstract—The influence of different reaction systems on α-chymotrypsin-catalyzed synthesis of eledoisin and LH-RH peptides from (7 + 4) and (5 + 5) fragments was investigated. The peptide yield was determined in the following systems: buffered aqueous media, frozen solutions, organic media, and cosolvent mixtures. The experimental set up was tailored to allow the screening of an array of conditions with minimum consumption of peptide fragments (2.1 and 2.5 mM). The best yields (22% yield for eledoisin and 68% yield for LH-RH) were obtained in buffered aqueous solutions. It was found that the choice of buffer had a strong influence on the peptide yield; boric-borate and ammonium acetate buffers at pH 9, gave the best results. In buffered aqueous systems, both syntheses were scaled up by using a 10-fold increase in fragment concentration (21 and 25 mM). Under these conditions the yields rose to 57% and 80% of eledoisin and LH-RH, respectively. Moreover, during the synthesis of eledoisin and in the presence of boric-borate buffer pH 9, the peptide precipitated from the reaction medium preventing a secondary hydrolysis and facilitating the in situ product purification.

Introduction

Enzymes have become important tools in the synthesis of peptides and new ways of using them for the formation of amide bonds keep emerging.[1] Enzymatic methods have several attractive features.[2] It is possible to carry out reactions under mild conditions, and the use of protecting groups can be minimized due to the high regiospecificity of enzymes. Also, since enzymatic reactions are stereospecific, stereoisomeric mixtures can be used as starting material to obtain pure diastereomeric compounds. These advantages make the use of enzyme technology particularly interesting in peptide fragment condensation. Solid-phase peptide synthesis[3] is a fast and convenient method for obtaining milligrams of peptides up to a length of about 50 amino acid residues. For long peptides it has some significant drawbacks: the yield decreases rapidly with the peptide length, and the product can be difficult to purify due to the micro-heterogeneity caused by deletions and truncated sequences of the peptide.[4] One attractive way of reducing these problems is to carry out convergent syntheses. Suitable peptide fragments can be conveniently prepared by solid-phase methods and subsequently coupled either chemically or enzymatically. Chemical coupling often requires fully protected fragments, which are usually poorly soluble and difficult to purify. In addition, epimerization is likely to take place when these methods are used. If the fragments are coupled enzymatically, soluble, easily purified unprotected fragments can be used, and the reaction proceeds without epimerization. The product mixtures obtained after enzymatic fragment condensation have fewer unwanted by-products than those obtained by chemical coupling, resulting in a more straightforward product purification.

Enzymatic condensation of peptide fragments is normally carried out under kinetic control.[5] In this case, a serine-or a cysteine-protease catalyzes the acyl-transfer from the C^{α} ester of a peptide to the N^{α} amino group of another peptide derivative. Owing to their broad selectivity, proteases are not ideal acyl-transferases.

Key words: Biologically active peptides; eledoisin; luteinising hormone releasing hormone (LH-RH); kinetically controlled fragment condensation; Cam esters; α-chymotrypsin; aqueous medium; inverse substrate; trypsin.
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Hydrolysis of the acyl-enzyme, secondary hydrolysis (hydrolysis of the product), and transpeptidations with the initial peptide derivatives can therefore occur.[5] To eliminate or minimize these disadvantages, the enzyme, substrate, and reaction medium can be modified to drive the synthetic process towards the desired product. The enzyme can be remodeled either chemically or genetically.[6] The leaving group of the acyl-donor can be remodeled in such a way that the enzyme reacts exclusively with the acyl-donor and not with the internal amide bonds of the fragments or the product.[7] Reducing the amount of water in the reaction medium may minimize undesirable hydrolytic side reactions.[8] Furthermore, modification of the reaction medium may lead to precipitation of the product, increasing its stability and facilitating its purification.[9]

Enzymatic fragment condensations have been reported before.[9,10] The conditions described in many of these procedures cannot be extrapolated to other peptides, and they have to be optimized for each particular case. The final success in devising a strategy depends on the number of options available to the peptide chemist. In this work, the experience gained in our laboratory on reaction system modifications for the enzymatic synthesis of peptides has been applied to protease-catalyzed condensation of peptide fragments. Two biologically active peptides of similar size, the undecapeptide eledoisin, Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2,[11] and the decapeptide LH-RH (luteinising hormone releasing hormone), Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2,[12] were chosen as target molecules. Kinetically controlled condensations were carried out in the following reaction systems: free enzyme in different aqueous buffers (liquid or frozen) or aqueous buffer/organic solvent-mixtures and immobilized enzyme in organic medium containing a low percentage of water. Moreover, in consideration of the high cost of peptide fragments a protocol to use minimum amounts of substrates was pursued.

Results and Discussion

Selecting fragments for coupling

Since eledoisin and LH-RH contain aromatic amino acid residues (phenylalanine, tryptophan, and tyrosine), the protease α-chymotrypsin was considered to be a suitable catalyst for fragment condensation. Its ability to hydrolyze peptides that have either phenylalanine, tyrosine or tryptophan in position P_1 (notation according to Schechter and Berger[13]) is well-known.[14] Due to the presence of both tryptophan and tyrosine in the molecule of LH-RH, two different reactions could be devised; either a 3 + 7 or a 5 + 5 condensation. In order to use fragments of equal size, we opted for the second (5 + 5) reaction (Figure 1). In the case of eledoisin, only a 7 + 4 condensation was suitable (Figure 2).

The approach selected for the α-chymotrypsin-catalyzed fragment condensation of eledoisin and LH-RH was the kinetically controlled synthesis using acyl-donor activated on the carboxyl function. We had found earlier that carboxamido methyl ester (Cam) (Figure 1), gave better results compared to alkyl and benzyl esters.[8,15] This ester has been used for the synthesis of LH-RH. Abrahmsén et al.[16] have proposed a modified carboxamido methyl ester, i.e. glycolylphenylalanine amide ester, 'CaPheNH_2' (Figure 2) as an even better substrate (higher k_{cat}/K_M). Both types of ester were used for the more elusive synthesis of eledoisin. They were prepared according to a solid-phase procedure implemented in our laboratory.[17]

Reactions in aqueous medium

Since peptide fragments are expensive reactants, we wanted to find systems in which their concentration and the excess of acyl-acceptor were kept to a minimum. In this way, an array of different conditions could be tested with minimum consumption of fragments and only the best reactions were scaled up. Solutions of the fragments were initially prepared in 50 mM Tris–HCl buffer pH 7.8, which has been shown to be suitable in α-chymotrypsin catalyzed synthesis of peptides.[18] Reactions were carried out at room temperature (25 °C) using free enzyme. We coupled fragments with initial concentrations of acyl-donor and acyl-acceptor as low as 2.1 and 2.5 mM in a total reaction volume of only 50 μL. The concentration of enzyme (0.2 μM) was tuned to obtain a reaction rate which allowed careful monitoring of the reaction and determination of the maximum concentration of product.

The two nucleophiles H-Gly-Leu-Arg-Pro-Gly-NH_2 and H-Ile-Gly-Leu-Met-NH_2 managed to compete with the water molecules for the acyl-enzyme as can be seen

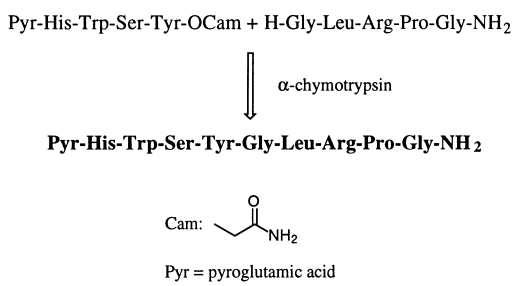

Figure 1. Reaction scheme for the enzymatic synthesis of LH-RH by fragment condensation (5 + 5).

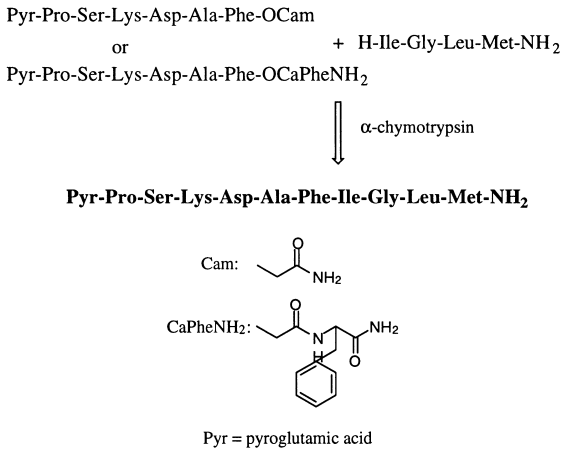

Figure 2. Reaction scheme for the enzymatic synthesis of eledoisin by fragment condensation (7 + 4).

in the chromatograms (Figure 3). The outcome of this competition, however, was different in the two syntheses. Eledoisin was synthesized in a 14% maximum yield, the hydrolyzed acyl-donor (Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OH) being the main product (85% yield). LH-RH was produced in a 68% maximum yield with only a 17% of acyl-donor ester hydrolysis by-product (Pyr-His-Trp-Ser-Tyr-OH). The higher yield of LH-RH could be due to the ionic interactions between α-chymotrypsin and arginine in P'_3.[19] The presence of leucine in P'_2 probably had an additional positive effect on the yield, since α-chymotrypsin has a preference for hydrophobic amino acid residues in this position.[20] It is noteworthy that the chymotryptic labile bond -Trp-Ser- of the acyl-donor was stable.

In an attempt to improve the yield in the synthesis of eledoisin, the $CaPheNH_2$ ester of the carboxyl terminal fragment (Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCa-Phe-NH_2) was used as an alternative acyl-donor. Under the same conditions described above, the yield obtained was not improved (14%). In any case, regardless of the final yield, both Cam and $CaPheNH_2$ esters provided high reaction rates. We suggest that both esters should be considered as potentially useful options for any fragment condensation reaction. In the present work, we have used them indiscriminately.

In order to increase the chemical yields, different modifications of the reaction medium have been tested. This includes changes in the reaction buffers, use of frozen solutions and use of organic solvents.

Different aqueous buffers as reaction medium

The choice of buffer and the pH may influence the performance of enzyme-catalyzed peptide synthesis

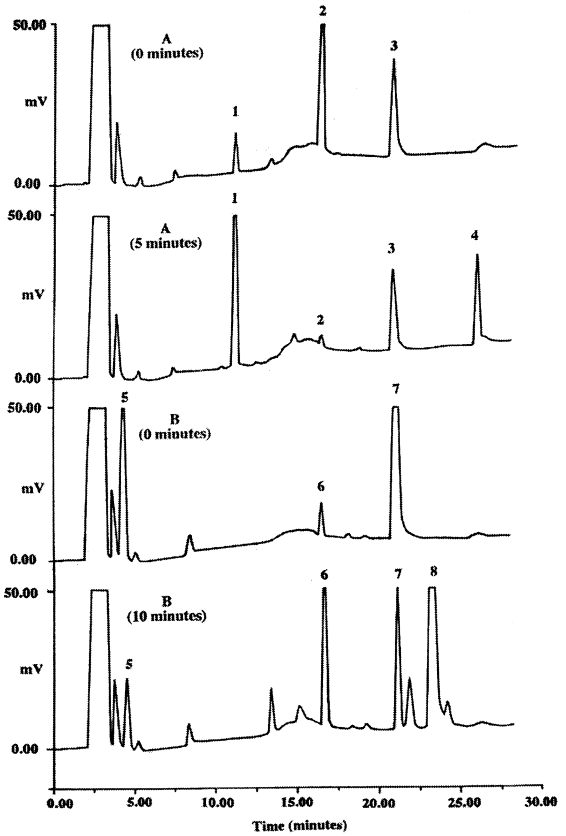

Figure 3. Synthesis of eledoisin (A) and LH-RH (B) by enzymatic fragment condensation in 50 mM Tris–HCl buffer pH 7.8. HPLC-profiles of the reaction mixtures are shown at the beginning and at the time of maximum product concentration (Table 1). Both syntheses were carried out at 25 °C in 50 μL of reaction mixture (50 μL for each reaction time), and they were catalyzed by α-chymotrypsin (0.2 μM). The initial concentrations of reactants were 2.1 mM acyl-donor (A: Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam and B: Pyr-His-Trp-Ser-Tyr-OCam) and 2.5 mM acyl-acceptor (A: H-Ile-Gly-Leu-Met-NH_2 and B: H-Gly-Leu-Arg-Pro-Gly-NH_2). Sample preparation: 15 μL of reaction mixture:acetic acid (1:1) were diluted to 100 μL with 0.1% aqueous trifluoroacetic acid. Load: 40 μL, column: Lichrocart 250-4 HPLC cartridge column (Lichrospher® 100, RP-18, 5 μm), elution: (A) TEAP pH 6.0 and (B) TEAP pH 6.0:acetonitrile (2:3), gradient: from 10 to 70% B in 30 min, flow rate: 1.0 μL/min, and detection: 215 nm. The numbers correspond to the following identified reactants and products: (1) Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OH, (2) Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam, (3) H-Ile-Gly-Leu-Met-NH_2, (4) eledoisin, (5) H-Gly-Leu-Arg-Pro-Gly-NH_2, (6) Pyr-His-Trp-Ser-Tyr-OH, (7) Pyr-His-Trp-Ser-Tyr-OCam, and (8) LH-RH.

reactions. In kinetically controlled synthesis, pH values above 7 are normally used in aqueous media. The buffer strength depends on the concentration of reactants, normally set around 50 mM. The pH of the reaction medium is usually corrected by the addition of NaOH

or HCl. In the present case, owing to the low reaction volume used (50 μL) it was not possible to adjust the pH by this procedure. Suitable buffers had to be chosen to make sure that the pH was stable at the given concentration of reactants and during the reaction time-course. The effect of different buffers on the yield of eledoisin was then investigated in aqueous solutions at room temperature (25 °C). The results obtained are summarized in Table 1. The following buffers were tested: 50 mM Tris–HCl (pH 7.8 and 9.0), 50 mM triethylamine-HCl pH 9.0, 50 mM ammonium acetate (pH 7.8 and 9.0), 250 mM tricine pH 7.8, 500 mM ammonium acetate pH 9.0, and 500 mM boric-borate pH 9.0. The most concentrated buffers (250 and 500 mM) were included to obtain information useful for scaling up purposes. To effectively control the pH, higher concentration of buffer salt is needed when higher concentrations of fragments are used. We wanted to test whether the enzymatic fragment condensation was affected in a negative way by increasing the concentration of buffer salts 5–10 times.

The yields of eledoisin ranged from 14 to 22% (Table 1). Boric-borate buffer (500 mM, pH 9.0) was the best one whereas ammonium acetate (50 mM, pH 7.8) and triethylamine-HCl (50 mM, pH 9.0) were not suitable for this fragment condensation: Hydrolysis of the ester was the only reaction that took place in ammonium acetate, and the enzyme was hardly active in triethylamine-HCl. A low effective concentration of the nucleophile due to insufficient deprotonation of H-Ile-Gly-Leu-Met-NH_2 could explain the low yield in 50 mM ammonium acetate pH 7.8. It is noteworthy that concentrated (250 and 500 mM) buffers were good reaction media. In the case of ammonium acetate pH 9.0, either 50 or 500 mM gave similar yields (20 and 17%).

Some of the buffers tested for eledoisin were applied to the synthesis of LH-RH: Tris–HCl (pH 7.8 and 9.0), ammonium acetate pH 9.0, and boric-borate pH 9.0 were used. The concentration in all cases was 50 mM. The results of these experiments are summarized in Table 1. The yields were similar in all the buffers tested. This fragment condensation gave a much higher yield of peptide (58–68%), for all the reaction media chosen than for the synthesis of eledoisin (14–22%).

Reactions in frozen solutions

It has been shown that peptides can be synthesized in frozen solutions, and the yields are sometimes considerably higher than at room temperature due to freeze concentration of the reactants.[21] Since the yield of eledoisin in 50 mM Tris–HCl buffer pH 7.8 was low (14%), freezing the reaction mixture was tried as an alternative way of promoting the fragment condensation. The results obtained are summarized in Table 2. The yield of the peptide was not increased, and the ester was still hydrolyzed to a significant extent. An additional peak appeared in the chromatograms (Figure 4A, peak 4) which was identified by mass spectrometry as corresponding to the deacylation of the acyl-enzyme by the Tris(tris(hydroxymethyl)-aminomethane) buffer (Pyr-Pro-Ser-Lys-Asp-Ala-Phe-NHC$(CH_2OH)_3$; ES-MS m/z: 878.3 $[(M+1)^+]$, $C_{39}H_{59}N_9O_{14}$ requires 878.0). The yield of this compound was high, ≈52% in 24 h. The corresponding Tris-adduct was also formed during the synthesis of LH-RH (data not shown). Undesirable interference of Tris in other reactions has been reported at room temperature at higher concentrations of Tris.[22]

Other buffers were tested for the synthesis of eledoisin. They include 50 mM ammonium acetate buffer pH 9.0

Table 1. Synthesis of eledoisin and LH-RH in aqueous media containing low concentrations of fragments[a]

Peptide	Buffer	Conversion (%)	Maximum yield (%)
Eledoisin	50 mM Tris–HCl pH 7.8	99	14
Eledoisin	50 mM Tris–HCl pH 9.0	99	19
Eledoisin	50 mM TEA-HCl pH 7.8	2	0
Eledoisin	50 mM CH_3COONH_4 pH 7.8	63	0
Eledoisin	50 mM CH_3COONH_4 pH 9.0	98	20
Eledoisin	500 mM CH_3COONH_4 pH 9.0	98	17
Eledoisin	250 mM tricine pH 7.8	98	17
Eledoisin	500 mM boric-borate pH 9.0	100	22
LH-RH	50 mM Tris–HCl pH 7.8	90	68[b]
LH-RH	50 mM Tris–HCl pH 9.0	92	65[b]
LH-RH	50 mM CH_3 $COONH_4$ pH 9.0	90	58[b]
LH-RH	50 mM boric-borate pH 9.0	90	61[b]

[a]The syntheses were carried out at 25 °C in 50 μL of aqueous buffer containing 2.1 mM acyl-donor, 2.5 mM acyl-acceptor, and 0.2 μM α-chymotrypsin. They were monitored for 10–30 min, and maximum yield and corresponding conversion were determined as described in Experimental.

[b]The yield of peptide passed through a maximum, which was reached before the acyl-donor was completely consumed.

Table 2. Synthesis of eledoisin in frozen aqueous media[a]

Buffer	C_{NaOH} (mM)	C_{enzyme} (μM)	Conversion (24 h) (%)	Yield (24 h) (%)
50 mM Tris–HCl pH 7.8	0	0.2	70	2
50 mM CH_3COONH_4 pH 9.0	0	1.0	99	11
50 mM boric-borate pH 9.0	0	1.0	5	2
NaOH (3.5 mM)	3.5	1.0	12	1
NaOH (4.8 mM)	4.8	2.0	46	1
NaOH (6.7 mM)	6.7	2.0	53 (10)[b]	10 (7)[b]

[a]The reactions were carried out at −30 °C in 50 μL of aqueous medium containing 2.1 mM Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam (acyl-donor), 2.5 mM H-Ile-Gly-Leu-Met-NH_2 (acyl-acceptor), and 0.2–2 μM α-chymotrypsin. Yield and conversion were determined as described in Experimental.
[b]Values in parentheses correspond to conversion and yield after 1 h.

and 50 mM boric-borate buffer pH 9.0. The results are included in Table 2. The combination of frozen solution and boric-borate buffer was not favorable due to the loss of enzymatic activity. The peptide was synthesized in 50 mM ammonium acetate pH 9.0, and the yield was similar to those obtained at room temperature. In this case, the chromatograms contained an additional peak (Figure 4B, peak 6). A mass analysis revealed that it was Pyr-Pro-Ser-Lys-Asp-Ala-Phe-NH_2 (ES-MS m/z: 774.9 $[(M+1)^+]$, $C_{35}H_{51}N_9O_{11}$ requires 773.8). The formation of this amide (≈50% yield in 24 h) seemed to be due to a reaction between the Cam ester and ammonia from the aqueous buffer.

Since the buffers appeared to react with the acyl-enzyme, sodium hydroxide was added instead of buffer to liberate the free base of the nucleophile. Low yields of peptide were obtained (Table 2), and no species other than the hydrolysis product Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OH was observed.

Our results are in agreement with those from other authors,[23] in suggesting that an increase in the yield is not a general phenomenon when the enzymatic reaction is carried out in ice.

Reactions at reduced water content: predominantly organic medium and aqueous/cosolvent mixtures

Organic solvents containing small amounts of water have proved to be useful systems for protease-catalyzed peptide bond formation. Since successful 2 + 3 and 5 + 4 fragment condensations in acetonitrile containing 4% (v/v) of aqueous buffer had been performed in our laboratory,[8,9] our intention was to synthesize eledoisin and LH-RH in a similar reaction medium. In the present case, we had to modify the reaction medium in order to solubilize the fragments. Acetonitrile:dimethylformamide (1:1) containing 4% of aqueous buffer and 1% (v/v) of triethylamine was chosen as a low water content system.[24] Triethylamine was added in order to generate the free base of the acyl-acceptors, H-Ile-Gly-Leu-Met-NH_2·TFA and H-Gly-Leu-Arg-Pro-Gly-NH_2·2TFA. α-Chymotrypsin deposited on Celite was used as catalyst. The enzymatic condensation of fragments was unsuccesful in both cases. Hydrolysis of the ester was the only reaction that took place (data not shown). Due to the limited solubility of the fragments in organic solvents, it was not considered worthwhile to further investigate the use of these systems.

It is known that the yield of peptide may increase upon addition of a cosolvent to an aqueous reaction medium. Barbas et al.[25] have reported a 200% increase of the yield of Bzl-Arg-Gly-NH_2 when the percentage of water was reduced from 100 to 40%. The higher yield in 60% dioxane was attributed to the elimination of the amidase activity of the enzyme. The authors showed that co-solvents such as acetonitrile and dimethylformamide (DMF) were also appropriate. We applied this approach to the synthesis of eledoisin using DMF as cosolvent. Dimethylformamide offered good solubility properties to both the fragments and the buffer salts used. Reactions were carried out in 50 mM Tris–HCl buffer pH 7.8 that contained 30, 40 or 50% (v/v) of organic solvent, using free α-chymotrypsin (2 μM) as catalyst. The effect of the organic solvent was unexpected: poor yields were obtained by increasing the DMF content; 6% (30% DMF), 4% (40% DMF), and 3% (50% DMF). In all cases, the ester was almost completely consumed (97–98%) when the maximum yield was reached. As suggested by other authors,[26] the effect of the organic solvent depends on the structure of reactants and the type of enzyme. Increasing the solvent concentration does not always favors the synthesis over the acyl-donor ester hydrolysis.

Cosolvent systems were also used in the synthesis of LH-RH. The effect of dimethylformamide (40, 50, and 70% (v/v)) on the peptide yield and the conversion of the acyl-donor is shown in Figure 5. It was possible to use concentrations of DMF as high as 50% with retention of enzymatic activity. Although no improvement was detected upon addition of DMF, its ability to

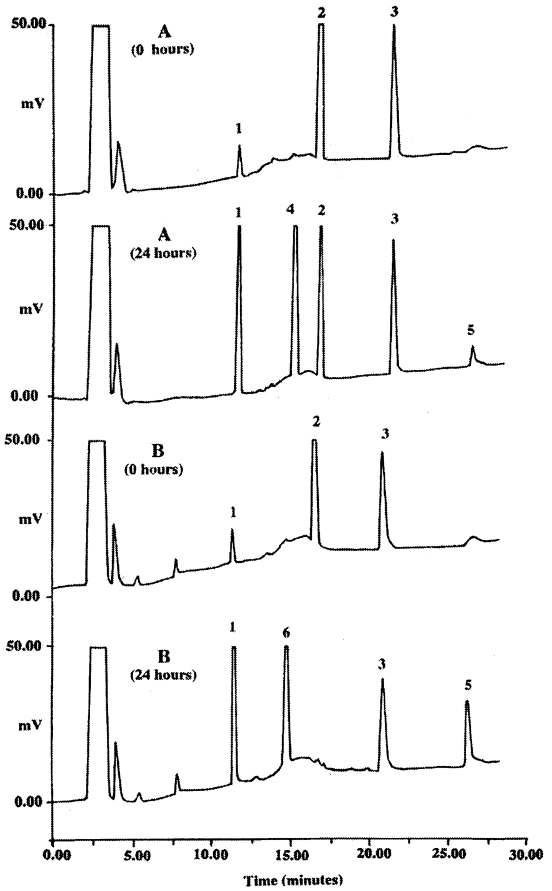

Figure 4. Synthesis of eledoisin in frozen solutions: (A) 50 mM Tris–HCl buffer pH 7.8 and (B) 50 mM ammonium acetate pH 9.0. HPLC-profiles of the reaction mixtures are shown at the beginning and after 24 hr (Table 2). The reaction conditions were the same as in Figure 3 except for the phase of the reaction mixture (frozen) and the concentration of enzyme in B (1 μM). The numbers correspond to the following identified reactants and products: (1) Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OH, (2) Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam, (3) HIle-Gly-Leu-Met-NH_2, (4) Pyr-Pro-Ser-Lys-Asp-Ala-Phe-NHC$(CH_2OH)_3$ (Tris-adduct), (5) eledoisin, and (6) Pyr-Pro-Ser-Lys-Asp-Ala-Phe-NH_2.

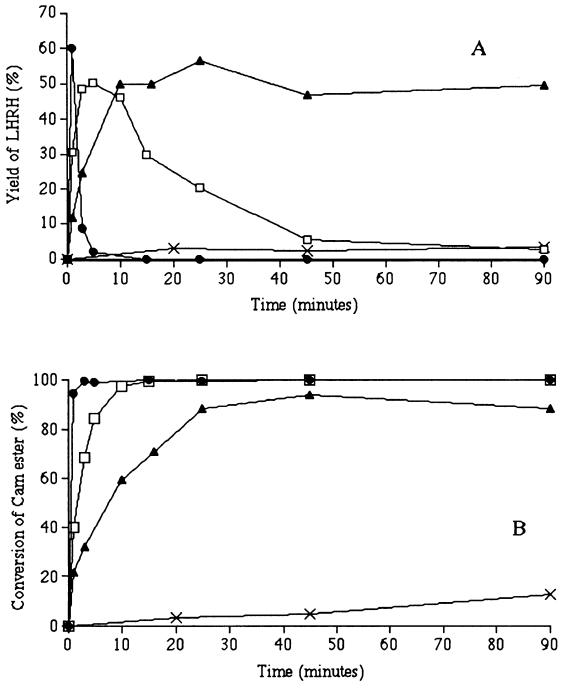

Figure 5. The time course of the synthesis of LH-RH in different mixtures of 50 mM Tris–HCl buffer pH 7.8 and dimethylformamide: (A) yield of peptide, (B) conversion of ester, (●) 100% aqueous buffer, (□) 60% aqueous buffer, (▲) 50% aqueous buffer, and (X) 30% aqueous buffer. The syntheses were carried out at room temperature in 50 μL of reaction mixture. Concentrations: 2.1 mM Pyr-His-Trp-Ser-Tyr-OCam, 2.5 mM H-Gly-Leu-Arg-Pro-Gly-NH_2, and 2 μM α-chymotrypsin.

facilitate peptide solubilization and the fact that this solvent is compatible with the enzyme activity could be useful in scale-up protocols.

Scale-up: reactions in aqueous media at high concentrations of reactants

When the best buffers had been found for each peptide, the condensation reactions were scaled up by increasing the concentration of the fragments and the concentration of buffer salts accordingly. Scale-up synthesis of eledoisin was performed at concentration of fragments of 21 and 25 mM (10-fold with respect to screening experiments). The buffers used were: 250 mM Tris–HCl pH 7.8, 250 mM tricine pH 7.8, 500 mM ammonium acetate pH 9.0, and 500 mM boric-borate pH 9.0. The results are summarized in Table 3. Boric-borate buffer 500 mM pH 9.0 gave the highest yield (57%) and 250 mM Tris–HCl buffer pH 7.8 gave the lowest yield (30%). Interestingly, both yields were higher than the ones obtained at low concentrations of fragments. It is noteworthy that the concentration of ammonium acetate could be increased from 50 to 500 mM without provoking a significant formation of the amide by-product Pyr-Pro-Ser-Lys-Asp-Ala-Phe-NH_2 (≈6%). The lower yield obtained in 250 mM Tris–HCl pH 7.8 was due to the formation of the Tris-adduct (≈16%), favored by the high concentration of Tris buffer. This side reaction had been observed previously in frozen 50 mM Tris–HCl buffer pH 7.8 (Figure 4A, peak 4).

Since the yield increased from 22 to 57% when the concentration of reactants was increased 10 times in the best buffer (500 mM boric-borate buffer pH 9.0), we raised the concentration even further (to 42 and

Table 3. Synthesis of eledoisin in aqueous media containing high concentrations of fragments[a]

Enzyme	C_{enzyme} (μM)	Buffer	$C_{reactants}$ (mM)[b]	Conversion (%)	Maximum yield (%)
α-CT	0.05	250 mM Tris-HCl pH 7.8	21:25	90	30
α-CT	0.05	250 mM tricine pH 7.8	21:25	93	40
α-CT	0.05	500 mM CH_3COONH_4 pH 9.0	21:25	99	48
T	0.05	500 mM CH_3COONH_4 pH 9.0	21:25	91	22
α-CT	0.05	500 mM boric-borate pH 9.0	21:25	99	57
α-CT	0.10	500 mM boric-borate pH 9.0	42:50[c]	99	58

[a]The reactions were carried out at 25 °C in 100 μL of aqueous medium containing 21–42 mM Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam (acyl-donor), 25–50 mM H-Ile-Gly-Leu-Met-NH_2 (acyl-acceptor) and 1.25 mg enzyme/L. They were monitored for 190–210 min by withdrawing samples of 5 μL at suitable time intervals. The time course of the reactions in 500 mM ammonium acetate buffer pH 9.0 are shown in Figure 6. Maximum yield and corresponding conversion were determined as described in Experimental. α-CT: α-Chymotrypsin; T: Trypsin.

[b]Acyl-donor and acyl-acceptor, respectively.

[c]Since the peptide precipitated, no samples were withdrawn, and the experiment was ended after 125 min by adding 200 μL of acetic acid to the reaction mixture.

50 mM). In this case, the yield of eledoisin was similar to the one obtained at concentrations of 21 and 25 mM (Table 3). Interestingly, the peptide formed precipitated during the reaction. When this happens, secondary hydrolysis is reduced, and the purification of the peptide is facilitated (in situ product recovery).

A similar study was conducted for the synthesis of LH-RH. In this case the concentration of fragments could not be increased 10 times due to the low solubility of the ester in 500 mM boric-borate buffer pH 9.0. This problem was circumvented by using 500 mM ammonium acetate-buffer pH 9.0/DMF (3:2) as reaction medium. As it was shown in the previous section and Figure 5, this reaction system was fully compatible with enzymatic activity. When the concentrations of fragments were increased 10 times (21 and 25 mM), a higher yield was obtained (80% versus 68%).

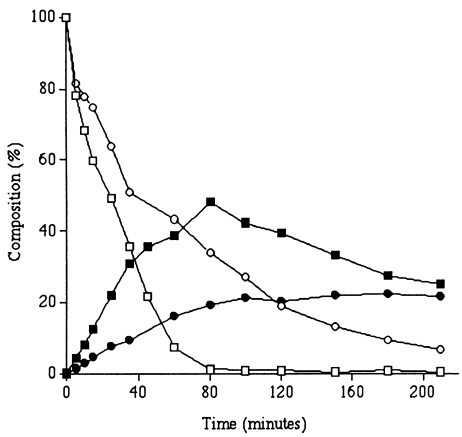

Figure 6. The time course of the synthesis of eledoisin catalyzed by different enzymes in aqueous medium: conversion (open symbols), yield of peptide (filled symbols), α-chymotrypsin (□, ■), and trypsin (○, ●). The reactions were carried out at 25 °C in 500 mM ammonium acetate buffer pH 9.0 (100 μL), and they were monitored by withdrawing samples of 5 μL at suitable time intervals. Concentrations: 21 mM Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam, 25 mM H-Ile-Gly-Leu-Met-NH_2, and 1.25 mg of enzyme/L.

Carboxamidomethyl ester (Cam) as inverse substrates for trypsin

Substrate engineering makes it possible to extend the primary specificity of an enzyme. Trypsin that normally accepts only lysine or arginine in P_1 can accept other amino acids that are not positively charged when certain ester groups are introduced into the molecule. Thus, when Cam esters are used as acyl-donors, it is possible to apply this so called inverse substrate strategy[27] using trypsin as catalyst. Its usefulness in fragment condensation was tested in the synthesis of eledoisin in 500 mM ammonium acetate buffer pH 9.0 (Figure 6). It was indeed feasible to synthesize this peptide, and a yield of 22% was obtained at 91% conversion. It must be emphasized that reduction of the yield when trypsin was used as catalyst was not caused by any new side reaction such as transpeptidation. It was due simply to a less favorable ratio of aminolysis to hydrolysis. It is noteworthy that the internal amide bond -Lys-Asp- of the acyl-donor ester was not cleaved by trypsin.

Conclusions

Cam esters can be used as acyl-donors in enzymatic fragment condensations in diluted solutions. This allows the screening of a great variety of reaction conditions with minimum consumption of valuable peptides. Thus, optimum reaction conditions can be used to scale up the

processes. This approach has been applied successfully to the 5 + 5 synthesis of LH-RH and the 7 + 4 synthesis of eledoisin. These two condensation reactions differ from simple dipeptide syntheses in terms of optimal reaction medium. Aqueous media were ideal for both peptides. Non-conventional media such as frozen solutions and low water content media have been found to be unsuitable for α-chymotrypsin-catalyzed fragment condensation, since the ratio of aminolysis to hydrolysis was not favorable. Organic solvents such as dimethylformamide can be added to the reaction medium to help to solubilize the fragments. Their function, in this case, was not the same as in dipeptide synthesis, in which they promote the aminolysis of the acyl-enzyme. This result is of interest when the reactions are to be scaled up by increasing the concentration of fragments as shown in the preparative synthesis of LH-RH. Interestingly, the condensation yields always increased when the concentration of fragments rose from 2.1 mM to 21 mM.

The results presented in this paper together with the fact that Cam esters can be obtained easily by solid-phase peptide synthesis protocols, makes their use a very promising approach for enzymatic peptide fragment condensation.

Experimental

Materials

α-Chymotrypsin type II (EC 3.4.21.1), trypsin type IX (EC 3.4.21.4), and *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine ('tricine') were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Technical-grade dichloromethane was obtained from Quimivita (Barcelona, Spain), and it was purified by distillation. Acetonitrile (HPLC-grade) was purchased from Labscan Ltd. (Dublin, Ireland). 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy-resin was obtained from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)-benzhydrylamine-resin and *N*-α-Fmoc-amino acid derivatives for solid phase peptide synthesis (Fmoc strategy) were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland) and Saxon Biochemicals GmbH (Hannover, Germany). Cesium carbonate was purchased from Merck (Darmstadt, Germany). Bromoacetic acid and 1,3-diisopropylcarbodiimide were obtained from Aldrich-Chemie (Steinheim, Germany). Tris(hydroxymethyl)aminomethane ('Tris') and 1-hydroxybenzotriazole were obtained from Fluka Chemie AG (Buchs, Switzerland). Trifluoroacetic acid (synthesis grade) was purchased from SDS s.a. (Peypin, France) and it was distilled before used in all HPLC-analyses. Celite (30–50 mesh) was purchased from Mandville (Pleasanton, CA, USA). De-ionized and Milli-Q grade water were used for preparative and analytical HPLC, respectively. All other solvents and chemicals used were of analytical grade.

Methods

Reversed-phase high performance liquid chromatography

Solid-phase products. HPLC analysis of synthesized peptide fragments was performed on a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC™ C_{18}, 5 μm, 0.46×25 cm column (The Separations Group, Hesperia, CA, USA). Preparative HPLC runs were performed on a Waters (Milford, MA, USA) Prep LC 4000 pumping system and a Waters PrePack® 1000 module fitted with a PrePack® (Waters) column (47×300 mm) filled with either VYDAC™ C_{18}, 300 Å, 15–20 μm stationary phase or Bondapack C_{18}, 300 Å, 15–20 μm stationary phase for the purification of LH-RH and eledoisin, respectively. Solvent systems and elution conditions are specified in 'Synthesis of fragments'.

Enzymatic reactions were followed by HPLC on a Merck-Hitachi Lichrograph system (Darmstadt, Germany) using a Lichrocart 250-4 HPLC cartridge, 250×4 mm, filled with Lichrospher® 100, RP-18, 5 μm (Merck). The following solvent system was used: (A) TEAP pH 6.0 and (B) TEAP pH 6.0:acetonitrile (2:3); gradient elution from 10 to 70% B in 30 minutes; flow rate 1.0 mL/min; detection 215 nm. Peaks of produced eledoisin and LH-RH were identified by comparing the retention times with authentic samples of eledoisin and LH-RH synthesized by solid phase methododology. Yields were determined by using an external standard method.

Mass spectrometry analysis

Molecular weights were determined by electrospray mass spectrometry (ES-MS) at the Servei d'Espectrometria de masses de la Universitat de Barcelona (fragments of eledoisin and by-products) and the Departament de Bioanalítica Mèdica, IIBB-CSIC (fragments of LH-RH).

Synthesis of fragments: Pyr-His-Trp-Ser-Tyr-OCam, H-Gly-Leu-Arg-Pro-Gly-NH_2, Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam, Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCaPhe-NH_2, and H-Ile-Gly-Leu-Met-NH_2. Fragments of eledoisin and LH-RH were manually prepared by solid-phase synthesis[28] on a 1 mmol scale applying the Fmoc-protocol. 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy-resin was used in all syntheses except in the synthesis of H-Ile-Gly-Leu-Met-NH_2, in which

Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)-benzhydrylamine-resin was used. All solvents that were used were of analytical grade (methanol and *N,N*-dimethylformamide) or were locally purified by distillation (dichloromethane). *N,N*-Dimethylformamide was dried and stored over 3 Å molecular sieves. It was flushed with nitrogen immediately before it was used in order to get rid of volatile amines. The first residues of the peptide esters Pyr-His-Trp-Ser-Tyr-OCam, Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam, and Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCaPhe-NH_2 were incorporated according to a solid phase procedure implemented in our laboratory for Cam esters.[17] Briefly, bromoacetic acid (4 equiv.) is linked to the resin using 1,3-diisopropylcarbodiimide as coupling reagent for 1 h. The first amino acid of the acyl-part of the ester is then coupled to the resin via the cesium salt method.[29] In the case of CaPheNH_2, the first step of the synthesis was the coupling of Fmoc-Phe-OH to the resin and the elimination of the N-protection using standard solid phase methods followed by the protocol described above.

Chain extension was accomplished by stepwise incorporation of the Fmoc-amino acids (2.5–5 equiv.) by 1,3-diisopropylcarbodiimide/1-hydroxybenzotriazole mediated couplings.

When the sequences were completed, test cleavages were carried out on 3 mg samples and monitored by HPLC. The following mixtures were found to be appropriate for treating the different resins: TFA/anisole/EDT/DCM (75/5/1/19): Pyr-His-Trp-Ser-Tyr-OCam, TFA/thioanisole/water/DCM (75/10/1/14): H-Gly-Leu-Arg-Pro-Gly-NH_2, TFA/anisole/EDT/DCM/water (75/5/3/16/1): H-Ile-Gly-Leu-Met-NH_2, TFA/anisole/water/DCM (85/5/1/9): Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam and Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCaPhe-NH_2.

Free peptides were obtained by treating the peptidyl-resins (1.8–3.0 g) with appropriate cleavage mixtures (28 ml) at 37 °C for a period of time ranging from 1 h 45 min to 3 h 30 min. The crude peptides were precipitated by adding cold tert-butyl methyl ether (70 mL). In the case of H-Ile-Gly-Ser-Met-NH_2, the mixture had to be placed in the freezer overnight to promote the precipitation. After centrifugation the pellet was washed with cold tert-butyl methyl ether (3×70 mL). The crude was dissolved in 0.1 aqueous TFA and the resin was filtered off. The solution was freed of ether at reduced pressure, and it was then lyophilized overnight.

Crude products were analyzed by HPLC on a VYDAC™ C_{18}, 5 μm, 4.6×250 mm column, eluted with a binary system, (A) 0.1% aqueous trifluoroacetic acid, (B) 0.08% trifluoroacetic acid in water:acetonitrile (2:3) under gradient conditions from 10 to 70% B over 30 min, at a flow rate of 1.5 mL/min, with detection at 215 nm.

The crude products were loaded onto a preparative PrePack® (Waters) column (47×300 mm) filled with either VYDAC™ C_{18}, 300 Å, 15–20 μm stationary phase for the purification of the LH-RH fragments or Bondapack C_{18}, 300 Å, 15–20 μm stationary phase for the purification of the eledoisin fragments. The peptides were purified by CH_3CN gradients in TEAP pH 2.3–2.4 (slope 0.2% CH_3CN/min) and desalted by fast CH_3CN gradients in 0.1% aqueous TFA. The flow rate was 100 mL/min and the products were detected at either 215 or 225 nm. Analysis of the fractions was accomplished under isocratic conditions in 0.1% aqueous TFA/CH_3CN using the same analytical column, flow rate, and detection as previously described. The final products were obtained by lyophilization as the trifluoroacetic salts of Pyr-His-Trp-Ser-Tyr-OCam (69 mg, ES-MS *m/z*: 760.6 $[(M+1)^+]$, $C_{36}H_{41}N_9O_{10}$ requires 759.8), H-Gly-Leu-Arg-Pro-Gly-NH_2 (385 mg, ES-MS *m/z*: 498.5 $[(M+1)^+]$, $C_{21}H_{39}N_9O_5$ requires 497.6), Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCam (109 mg, ES-MS *m/z*: 832.5 $[(M+1)^+]$, $C_{37}H_{53}N_9O_{13}$ requires 831.9), Pyr-Pro-Ser-Lys-Asp-Ala-Phe-OCaPheNH_2, (62 mg, ES-MS *m/z*: 979.5 $[(M+1)^+]$, $C_{46}H_{62}N_{10}O_{14}$ requires 979.1), and H-Ile-Gly-Leu-Met-NH_2 (240 mg, ES-MS *m/z*: 431.7 $[(M+1)^+]$, $C_{19}H_{37}N_5O_4S_1$ requires 431.6).

Enzyme immobilization

The enzyme was deposited on a solid support. A solution of α-chymotrypsin (63.6 mg in 2 mL of 50 mM Tris–HCl buffer pH 7.8) was mixed with celite (2 g), and the preparation was dried under vacuum.

Enzymatic reactions

Reactions in aqueous media at low concentrations of fragments. Eledoisin and LH-RH were synthesized from fragments (obtained by solid-phase synthesis) in different aqueous buffers or mixtures of 50 mM Tris–HCl buffer pH 7.8 and dimethylformamide, 0–70% of DMF (v/v). All reactions were carried out at 25 °C in 1.5 mL Eppendorf tubes that contained 50 μL of reaction mixture: acyl-donor (Cam ester or CaPheNH_2 ester) (2.1 mM), acyl-acceptor (a peptide amide) (2.5 mM), and α-chymotrypsin (0.2–2.0 μM). The reaction mixtures were prepared in the following way: The peptide fragments were dissolved in pure water (1 mL) (acyl-donor: 2.3 mM and nucleophile: 2.8 mM), and aliquoted (45 μL fractions) in Eppendorf tubes. Then, the water was evaporated at reduced pressure. The residue was dissolved in 45 μL of either aqueous buffer or 50 mM Tris–HCl buffer pH 7.8 containing dimethylformamide (0–77.8% v/v). The enzymatic reaction was

started by adding the enzyme (5 μl, 2–20 μM α-chymotrypsin in the same buffer). The enzymatic reaction was stopped by adding 50 μL of acetic acid. Since no samples were withdrawn, several tubes had to be used for each synthesis in order to monitor the reaction. Two zero samples were prepared for each reaction in the following way: Acetic acid (50 μL) and enzyme solution (5 μL) were added to the solution of peptide fragments (45 μL). Samples for HPLC-analysis were prepared by diluting 15 μL of reaction mixture:acetic acid (1:1) to 100 μL with 0.1% aqueous trifluoroacetic acid.

Reactions in frozen solutions. Reactions were carried out at −30 °C in 1.5 mL Eppendorf tubes containing 50 μL of reaction mixture. Several tubes were used for each reaction time in order to monitor the synthesis. Solutions of peptide fragments in aqueous buffers (45 μL) were prepared in the same way as for the reactions at room temperature (previous paragraph). Reactions in the presence of aqueous sodium hydroxide were carried out as follows. The acyl-donor (2.3 mM) was dissolved in water (1 mL), and aliquoted (45 μL fractions) in Eppendorf tubes. The water was evaporated at reduced pressure, and a solution (45 μL) of the acyl-acceptor (2.8 mM) in aqueous sodium hydroxide (3.9–7.4 mM) was added.

The solution of fragments (45 μL) were preincubated at 0 °C (in ice). The syntheses were then started by adding α-chymotrypsin (5 μL of 2–20 μM), rapidly mixing the solution, and placing the tube in ethanol/solid carbon dioxide (−78 °C) for half a minute. The enzymatic reaction was then continued in a freezer at −30 °C. The reactions were stopped by adding 50 μL of acetic acid and melting the frozen solution at 25 °C (1 min). The zero and HPLC samples were prepared as previously described.

Reactions at reduced water contents. A solution (1 mL) of acyl-donor (2.1 mM) and acyl-acceptor (2.5 mM) was prepared by dissolving the fragments in dimethylformamide:acetonitrile (1:1) that contained Tris–HCl buffer 50 mM, pH 7.8 (4% v/v) and triethylamine (1% v/v). The enzymatic reaction was started by adding α-chymotrypsin deposited on celite (75 mg, 31.8 mg of enzyme/g of support). The vial was then placed horizontally in a shaker (180 rpm) at 25 °C. The reaction was monitored for 48 h by withdrawing samples of 15 μL. Each sample was mixed with 15 μL of acetic acid and diluted to 200 μL with aqueous 0.1% trifluoroacetic acid and subsequently analyzed by HPLC.

Reactions in aqueous media at high concentration of reactants. The fragments of eledoisin (acyl-donor: 2.2–4.4 mg and acyl-acceptor: 1.5–3.0 mg) were dissolved in 100 μL of aqueous buffer (Table 3). The fragments of LH-RH (2.0 mg of both acyl-donor and acyl-acceptor) were dissolved in 100 μL of 500 mM ammonium acetate buffer pH 9.0:dimethylformamide (111:89). Two zero samples (2×5 μL) were withdrawn and mixed with 10 μL of acetic acid. The condensation was started by adding the enzyme solution (10 μL, 0.5–20 μM α-chymotrypsin). When the enzyme was added, the reaction mixture had the following composition: 21–42 mM acyl-donor, 25–50 mM acyl-acceptor, and 0.05–2 μM α-chymotrypsin. In the case of LH-RH the final concentration of dimethylformamide was 40%. The syntheses were monitored by withdrawing samples of 5 μL. Each sample was added to 10 μL of acetic acid in an HPLC-vial to stop the enzymatic reaction. They were then diluted with 0.1% aqueous trifluoroacetic acid to 720 μL and analyzed by HPLC.

In the case of eledoisin, it was not possible to withdraw samples to monitor the reaction when the concentration of reactants was increased to 42 and 50 mM since the product precipitated. A suitable reaction time was therefore determined, and the experiment was repeated. The only samples that were withdrawn this time were the two zero-samples. Acetic acid (200 μL) was added after 125 mins to stop the reaction and dissolve the precipitate. A sample for HPLC-analysis was then prepared by diluting 15 μL of reaction mixture/acetic acid (1:2) to 740 μL by adding 0.1% aqueous trifluoroacetic acid.

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