In Vitro Amidation for the Preparation of an α-Amidated Peptide: Enzymatic Coupling with Prolyl Endopeptidase

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By enzymatic coupling with an endopeptidase specific for proline residue, *i.e.*, prolyl endopeptidase, an α-amidated peptide (LH-RH) has been prepared from its acid form precursor without any byproduct.

Many biologically active peptides have the α-amide structure at their *C*-termini and in most cases the amide structure is essential for their biological activities.[1] These amidated peptides cannot be directly produced with microorganisms by means of recombinant DNA, since microbes lack the function of the post-translational modification to form the *C*-terminus amide. The free-acid form of peptides expressed in *Escherichia coli* or yeast is thus to be converted to the amide form by an *in vitro* amidation process.[2]

The enzymatic reaction with peptidylglycine α-amidating monooxygenase (PAM)[3] has been known as the sole way of *in vitro* amidation that converts unprotected peptides to the amide form in satisfactory yields. The preparation of PAM, however, requires animal-cell culture or extraction from horse serum, the cost of which is prohibitive for its use in a production process. The enzymatic coupling of an amino acid amide (AA–NH_2) to the *C*-terminus of a precursor peptide is also known as an alternative method of *in vitro* amidation.[4] Nevertheless, non-selective hydrolytic cleavage and transpeptidation, which are due to broad specificities of the peptidases in common use, are very often associated with the coupling reaction to result in the formation of byproducts and low yields of the desired coupling products. To overcome this drawback of the conventional enzymatic coupling we have examined the use of specific endopeptidases rather than the peptidases with broad specificities. A variety of specific peptidases are now commercially available and the variation enables us to select a peptidase with an appropriate substrate specificity. We report here prolyl endopeptidase (PEP) was successfully applied for the *in vitro* amidation of an acid-form precursor of luteinizing hormone releasing hormone (LH-RH), which is a decapeptide currently used as a human pharmaceutical and for *in vitro* diagnosis.

PEP is an endopeptidase that cleaves a peptide specifically at the *C*-terminus side of a proline residue. Since LH-RH has proline residue at the penultimate position in the amino-acid sequence, it is subjected to specific cleavage with PEP to give a large *N*-terminus fragment terminated with the proline residue and glycine-amide (Gly–NH_2). We prepared LH-RH by the reverse manner of the hydrolytic cleavage, *i.e.* an enzymatic coupling by condensation. Gly–NH_2 was coupled to the *N*-terminal nonapeptide, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro [LH-RH(1–9)] with recombinant PEP, which we cloned from *Flavobacterium meningosepticum*[5] and expressed in *E. coli* [eqn. (1)]. As a general procedure of the coupling reaction, 1 mmol dm^{-3} of the substrate fragment was incubated with Gly–NH_2 in the presence of 0.08 μmol dm^{-3} PEP at 30 °C. Aliquots of the reaction mixture were withdrawn at various intervals and subjected to HPLC analysis. The reaction proceeded promptly even with PEP in such a high substrate : enzyme ratio as 12 500 : 1 to give LH-RH, but the rate of the LH-RH formation gradually dropped as the substrate was consumed. In about 6 h the coupling reaction appeared to approach equilibrium with the reverse reaction, *i.e.*, the hydrolysis, and a steady state between LH-RH and the substrate was attained by 24 h. No product formation was observed without PEP.

$$\text{<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro} + \text{Gly-}NH_2 \overset{\text{PEP}}{\rightleftharpoons} \underset{\text{LH-RH}}{\text{<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-}NH_2} \quad (1)$$

The conversion of the precursor to LH-RH after reaching apparent equilibrium was found to be largely dependent on the concentration of Gly–NH_2 and pH of the reaction mixture. The conversion increased with an increase in the concentration of Gly–NH_2 and reached the maximal value with 2.5 mol dm^{-3} Gly–NH_2 [Fig. 1(*a*)]. Further increase of the concentration of Gly–NH_2 led the gradual decrease in the

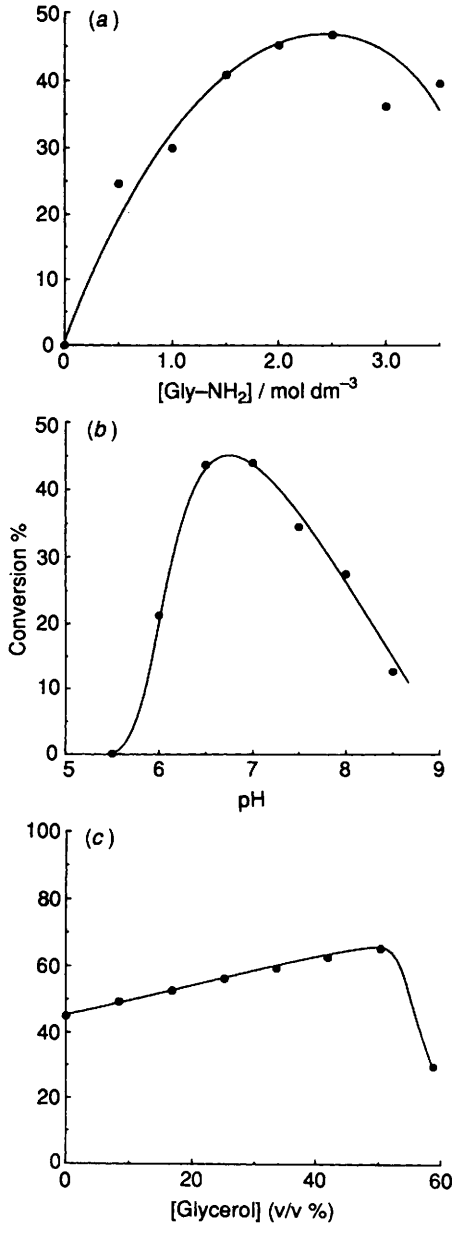

Fig. 1 Effects of (*a*) concentration of Gly–NH_2, (*b*) pH and (*c*) concentration of glycerol on conversion of a precursor peptide [LH-RH(1–9)] to the mature form in the coupling reaction with Gly–NH_2. *Reaction conditions*: 1 mmol dm^{-3} LH-RH(1–9), 0.08 μmol dm^{-3} PEP at 30 °C; (*a*) pH 7.0 for 24 h; (*b*) 2.0 mol dm^{-3} Gly–NH_2 for 6 h; (*c*) 2.0 mol dm^{-3} Gly–NH_2 at pH 7.0 for 24 h.

conversion, probably because of substrate/product inhibition of PEP. Fig. 1(*b*) shows the pH-conversion profile, indicating the optimal pH for the coupling reaction was 6.5–7.0, which is very close to the optimal pH for hydrolysis catalysed by PEP. With 2.5 mol dm^{-3} Gly–NH_2 at pH 7.0 the maximum conversion of 45% was attained.

To improve the conversion by shifting the equilibrium between the condensation and the hydrolysis, a water-miscible organic solvent was added to the reaction mixture. Among the examined solvents; 1,4-dioxane, DMF, DMSO, ethanol, glycerol and ethylene glycol, glycerol was most effective for improving the conversion of the PEP-catalysed coupling reaction. With the concentration of glycerol the conversion increased linearly and reached 67% at 50% (v/v) glycerol, and decreased rapidly beyond that concentration [Fig. 1(*c*)]. Ethylene glycol showed a marginal improvement in the conversion, while the rest of the solvents inhibited PEP even at 10% (v/v) and the coupling reaction stopped before reaching the equilibrium state. PEP is rather sensitive to the organic solvents that are commonly used in enzymatic coupling reactions.

PEP catalysed the coupling so selectively that no side product was detected in the HPLC analysis of the reaction mixture (Fig. 2). Since the unreacted substrate was easily recovered in the purification of the produced LH-RH, the chemical yield of LH-RH was quantitative on the basis of the consumed substrate. We confirmed the quantitative yield not only by the HPLC analysis but also by isolating the product and recovering the substrate from the reaction mixture. The precursor (11.9 μmol) was dissolved in 2.2 mol dm^{-3} Gly–NH_2 (2.14 cm^3, pH 7.0) containing 56% (v/v) glycerol, and PEP (0.24 cm^3 of a 0.8 μmol solution, 0.19 nmol) was added to the substrate solution. The reaction mixture was incubated at 30 °C for 72 h and subjected to purification by HPLC to give 6.46 μmol of LH-RH and 5.26 μmol of the substrate. Though the conversion (56%) was slightly lower because of a high [substrate]:[enzyme] ratio (62 000:1), excellent isolation yields were obtained both for LH-RH (97%) and the recovered substrate (>99%).

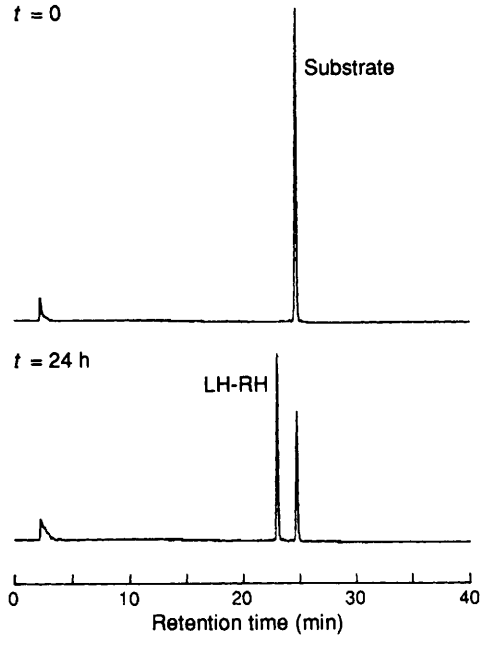

Fig. 2 HPLC analysis of the coupling reaction of LH-RH(1–9) with Gly–NH_2. *Conditions for* HPLC: column, YMC-Pack ODS-AM AM-302 (4.6 × 150 mm); mobile phase A, 0.1% TFA in 5% (v/v) MeCN; mobile phase B, 0.1% TFA in 55% (v/v) MeCN; gradient, 10–90%B in 40 min; flow rate, 1 cm^3 min^{-1}; detection at 280 nm.

Most enzymatic coupling reactions require the use of a peptidase in a relatively low [substrate]:[enzyme] ratio (10–100:1), which results in a high cost of the peptidase and inhibits a practical application of the reactions to preparation of the α-amidated peptide. The coupling reaction by use of the specific endopeptidase, as demonstrated above, allows a high [substrate]:[enzyme] ratio (13 000–62 000:1) and yet gives no byproduct. The excellent selectivity of the PEP catalysed coupling makes this *in vitro* amidation process very advantageous, because the purification of the product, as well as the recovery of the precursor peptide, can be easily performed even in a large production process. To complete the whole production process of recombinant LH-RH, the study to produce the acid-form precursor by recombinant DNA technology is under way.

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