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Short Communication

Highly efficient synthesis of endomorphin-2 under thermodynamic control catalyzed by organic solvent stable proteases with in situ product removal

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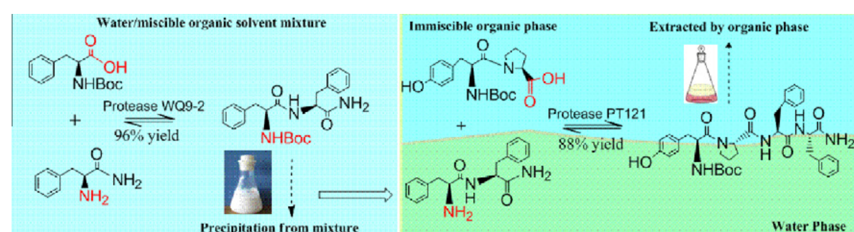
GRAPHICAL ABSTRACT



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ABSTRACT

An efficient enzymatic synthesis of endomorphin-2 (EM-2) was achieved using organic solvent stable proteases in nonaqeous media, based on thermodynamic control and an in situ product removal methodology. The high stability of biocatalysts in organic solvents enabled the aleatoric modulation of the nonaqueous reaction media to shift thermodynamic equilibrium toward synthesis. Peptide Boc-Phe-Phe-NH_2 was synthesized with a high yield of 96% by the solvent stable protease WQ9-2 in monophase medium with an economical molar ratio of the substrate of 1:1. The tetrapeptide Boc-Tyr-Pro-Phe-Phe-NH_2 was synthesized with a yield of 88% by another organic solvent tolerant protease PT121 from Boc-Tyr-Pro-OH and Phe-Phe-NH_2 in an organic–aqueous biphasic system. The reaction–separation coupling in both enzymatic processes provides “driving forces” for the synthetic reactions and gives a high yield and high productivity without purification of the intermediate, thereby making the synthesis more amenable to scale-up.

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1. Introduction

Over the past ten years, scientific and technological advances have established biocatalysis as a practical and environmentally friendly alternative to traditional metallo- and organocatalysis in chemical synthesis, both in the laboratory and on an industrial scale (Bornscheuer et al., 2012; Fernández-Lucas et al., 2012). Proteases are effective biocatalysts for the synthesis of peptides under nonaqueous conditions and give a more selective process, in turn preventing the formation of by-products (Bordusa, 2002; Gupta and Khare, 2006). The stereospecificity of proteases guarantees the formation of stereochemically uniform products and only semi-permanent protection of the carboxy and amino in Cα backbone would be required.

Endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2) has recently drawn much attention owing to the distinct medicinal values of this compound. It can relieve severe pain through activation of μ-opioid receptors, being more effective than endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and has shown great promise as analgesics of comparable potency to morphine (Liu and Wang, 2012). In addition, EM-2 can effectively protect neurons against biological effects

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induced by amyloid protein aggregation (mainly Aβ1-42), thus being exploited as a lead sequence for drug development in the treatment of Alzheimer's disease (Granic et al., 2010). For the synthesis of endomorphin-2 and its analogs, several chemical methods have been investigated, each of which has some drawbacks, such as the requirement of the protection of Tyr phenolic hydroxyl group during the solid phase synthesis and relatively complex intermediate product purification protocols during the liquid phase condensation (Biondi et al., 2006; Shi et al., 2007), thus limiting its industrial production.

An alternative to the traditional synthetic route, with a great potential of yielding a high purity product lies in the use of biocatalysis. For the enzymatic synthesis of peptides, both thermodynamically and kinetically controlled approaches have been developed. An activated ester, which is an acyl donor, is generally needed for the kinetically controlled syntheses (Salam et al., 2006). Alternatively, the use of a thermodynamically controlled approach might be of interest, as in this case, activation of the acyl donor is not required and no side products are formed other than water. In spite of this, as the reverse of the hydrolytic reaction, the low reaction rates, low product yield attainable and an excess needing of the amino components during the thermodynamically controlled synthesis limit its application (Bordusa, 2002). Actually, two principal ways can be adopted to shift the thermodynamic equilibrium toward peptide bonds formation: (1) increasing the concentration of uncharged substrates by alteration of their pK values; (2) increasing the accumulation of the peptide product based on the mass transfer of the product in the reaction medium (Jakubke et al., 1985; Tufvesson et al., 2011). Both can be achieved through the addition of organic solvent to the reaction medium. On the other hand, the addition of organic solvent dramatically increases the solubility of the substrates to keep their concentration at a high level. In this study, an enzymatic and scalable production route to produce bioactive EM-2 was reported in nonaqueous systems for the first time. Due to the toxicity of organic solvent to most enzymes, two previously reported organic solvent stable proteases from *Bacillus cereus* WQ9-2 and *Pseudomonas aeruginosa* PT121 were adopted (Rahman et al., 2005; Tang et al., 2008; Xu et al., 2010). They have shown a broad catalytic specificity for carboxylic acid residues and superior performances in the synthesis of endomorphin-1(Sun et al., 2011). The efficient synthesis was achieved through the coupling of reaction with the precipitation/extraction of products in two aqueous-organic solvent reaction media. The mechanism for the high efficiency was preliminary explored from a thermodynamic point of view here.

2. Methods

2.1. Biological and chemical materials

N-tert-Butoxycarbonyl-L-tyrosine (Boc-Tyr), N-tert-Butoxycarbonyl-L-phenylalanine (Boc-Phe), L-proline methyl ester hydrochloride (Pro-OMe·HCl), L-phenylaninamide (Phe-NH_2) and trifluoroacetic acid were purchased from GL Biochem (Shanghai, China). Boc-Tyr-Pro-OH was synthesized by ourself according to the method described in Supplementary material. Organic solvents (all HPLC grade) were purchased from Sinopharm (Shanghai, China). All other chemicals were of analytical grade and purchased from Sunshine (Nanjing, China).

The preparation of purified organic solvent tolerant proteases WQ9-2 and PT121, from *B. cereus* WQ9-2 and *P. aeruginosa* PT121, respectively, were as described in our previous reports (Tang et al., 2010; Xu et al., 2010). The strains of *B. cereus* WQ9-2 and *P. aeruginosa* PT121 are currently deposited at the China Center for Type Culture Collection (Wuhan, China) with accession numbers of CCTCCM2010010 and CCTCCM208029, respectively.

2.2. Enzymatic synthesis of Boc-Phe-Phe-NH_2

Boc-Phe (2.4 mmol) and Phe-NH_2 (2.4 mmol) were dissolved in 6 ml different organic solvents, followed by the addition of 14 ml of protease WQ9-2 (final concentration of 2400 U/ml, dissolved in 50 mM Tris–HCl with pH 8.0). The reaction mixture was incubated at 37 °C for 12 h. The yields of product were detected by HPLC (retention time: 26.7 min). The synthesized Boc-Phe-Phe-NH_2 was confirmed by the mass spectrometry {ESIMS: 434.5 (M+Na^+)}. The removal of the Boc group was described in Supplementary material.

2.3. Enzymatic synthesis of Boc-Tyr-Pro-Phe-Phe-NH_2

Boc-Tyr-Pro-OH (0.12 mmol) and Phe-Phe-NH_2 (0.24 mmol) were dissolved in 4 ml water-saturated organic solvent (listed in Table 1). Then, 2 ml of protease PT121 (15,000 U/ml, dissolved in 50 mM Tris–HCl with pH 7.5) was added, and incubated with shaking at 37 °C and 200 rpm for 10 h. At the end of the reaction, the organic layer was separated and washed with 1 M $NaHCO_3$, 10% (w/v) citric acid and a saturated NaCl solution, and then dried with anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure gave the crude yellow product. The synthesized Boc-Tyr-Pro-Phe-Phe-NH_2 was confirmed by mass spectrometry {ESIMS: 694.8(M+Na^+)}. The HPLC retention time was 17.2 min.

2.4. pK values determination

Ten millimole of each compound were dissolved in water or in water-cosolvent mixtures and titration was performed by using an automatic titrator with a combined electrode. The pK of carboxy group was performed by increasing the pH with 100 mM NaOH as titrating solution. The pH of solution containing amino group donor was previously adjusted to around 10.0 with dilute NaOH solutions, and then titration was performed by lowering the pH with 100 mM HCl as titrating solution. No corrections of pH values were made in the presence of organic solvents, and pK values were calculated as pK_{app}.

3. Results and discussion

3.1. Effect of hydrophilic organic solvents on the synthesis of Boc-Phe-Phe-NH_2 in organic-aqueous monophasic system

The C-terminal protected dipeptide precursor Boc-Phe-Phe-NH_2 was efficiently synthesized by protease WQ9-2 catalyzed coupling of Boc-Phe-OH with H-Phe-NH_2 in a water-miscible organic solvent system. Taking into account the pK_a value of the amino group (9.5) and the optimal activity of protease WQ9-2 (Xu et al., 2010), a pH 8.0 buffer was selected to ensure that the uncharged form of the amine was present in high concentrations. The reactants were used at a high concentration of 400 mM with an economical equimolar ratio. The condensation reaction was initiated by the addition of protease WQ9-2. The effects of different organic solvents and their concentrations on the yield of Boc-Phe-Phe-NH_2 are shown in Fig. 1. The desired product was obtained in high yield when catalyzed by protease WQ9-2 in 10–40% DMSO or DMF, and precipitated from these liquid systems. The product crystallized from 30% DMSO in 96% molar yield within only 10 min of reaction initiation as confirmed by HPLC analysis. The direct and quick crystallization of Boc-Phe-Phe-NH_2 from the medium greatly simplified the purification process. H-Phe-Phe-NH_2 was then generated by the removal of the Boc group using trifluoroacetic acid (TFA) with a yield of 91%.

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