Green Chemistry

Cite this: *Green Chem.*, 2011, **13**, 1680

www.rsc.org/greenchem

PAPER

Efficient chemo-enzymatic synthesis of endomorphin-1 using organic solvent stable proteases to green the synthesis of the peptide†

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Received 12th January 2011, Accepted 15th March 2011
DOI: 10.1039/c1gc15042a

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM-1), an effective analgesic, was efficiently synthesized by a combination of enzymatic and chemical methods. Peptide Boc-Trp-Phe-NH_2 was synthesized with a high yield of 97.1% by the solvent-stable protease WQ9-2 in a 20% methanol medium. The maximum concentration (141 g L^{-1}) of Boc-Trp-Phe-NH_2 was obtained with an economical molar ratio of the substrate of 1 : 1. The products crystallized and separated from the substrates without purification, followed by the removal of the Boc group with trifluoroacetic acid to generate Trp-Phe-NH_2. Using the efficient mixed carbonic anhydride method, Boc-Tyr-Pro-OH was synthesized chemically. The tetrapeptide Boc-Tyr-Pro-Trp-Phe-NH_2 was synthesized with a yield of 84.5% by another organic solvent-tolerant protease, PT121, from Boc-Tyr-Pro-OH and Trp-Phe-NH_2 in an organic–aqueous biphasic system and was extracted with ethyl acetate, shifting the equilibrium of the synthesis. EM-1 was obtained by removal of the Boc group from Boc-Tyr-Pro-Trp-Phe-NH_2 in a high yield of 91% due to the free protection of the side-chain of the Tyr phenolic hydroxy group. After a one-step purification in the final step by using high speed countercurrent chromatography (HSCCC), EM-1 was obtained with a purity greater than 99.8%. The chemo-enzymatic synthesis of EM-1 proved to be efficient, productive, with minimal side-chain protection and simple purification, thus greening the synthesis of the peptide.

Introduction

Morphine is the pharmacologically-active component of opium, a poppy plant extract that has been widely used for the relief of severe pain, although it is known to have a number of well known side effects, such as physical dependence, tolerance and respiratory depression.[1] In 1997, two opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), were isolated from bovine brain and demonstrated to have a high affinity for the μ-opioid receptor.[2] Endomorphins have shown great promise as analgesics of comparable potency to morphine, which has encouraged the use of natural or synthetic peptides as analgesics instead of morphine itself.[3–6]

Recently, endomorphins and their analogues have been synthesized, mainly by chemical methods, with protection of the Tyr phenolic hydroxy group.[7–12] Each of the known chemical methods for the preparation of endomorphins and their analogues have been reported to have some drawbacks. Besides the relatively low yields, low concentration, and the hazardous and expensive condensing reagents, all the reactive amino acid side chain functionalities have to be protected to prevent side reactions. However, chemo-enzymatic methods are attractive alternatives, because enzymatic reactions are regio- and stereoselective, and require minimal side-chain protection. Only one report has given an account of the enzymatic synthesis of endomorphin-1, where the partial precursor Boc-Trp-Phe-NH_2 and endomorphin-1 precursor Boc-Tyr-Pro-Trp-Phe-NH_2 were synthesized by an enzymatic method using immobilized papain with low yields of 27.8 and 35.2%, respectively.[13]

In this study, a chemo-enzymatic procedure for the synthesis of endomorphin-1 using solvent-stable proteases is described with much improved green chemistry characteristics. The strategy of endomorphin-1 synthesis is showed in Fig. 1. The reaction separation coupling of Boc-Trp-Phe-NH_2 and Boc-Tyr-Pro-Trp-Phe-NH_2 in the two enzymatic approaches gave a high yield and high productivity without purification of the intermediate, based on the high stability in solvents and favorable substrate specificity of proteases WQ9-2[14] and PT121.[15]

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c1gc15042a

Table 1 Catalysis specificity of protease WQ9-2 and PT121 in 50% (v/v) of various organic solvents

WQ9-2 protease			PT121 protease		
H-AA-Phe-NH_2	Organic solvent	Yield (%)	H-AA-Phe-NH_2	Organic solvent	Yield (%)
Cbz-Asp-Phe-NH_2	DMSO	54	Cbz-Asp-Phe-NH_2	DMSO	91
	Methanol	23		Methanol	52
	DMF	16		DMF	75
Cbz-Leu-Phe-NH_2	DMSO	8	Cbz-Leu-Phe-NH_2	DMSO	53
	Methanol	30		Methanol	44
	DMF	0		DMF	0
Cbz-Ala-Phe-NH_2	DMSO	75	Cbz-Ala-Phe-NH_2	DMSO	55
	Methanol	78		Methanol	58
	DMF	35		DMF	29
Cbz-Phe-Phe-NH_2	DMSO	86	Cbz-Phe-Phe-NH_2	DMSO	55
	Methanol	67		Methanol	65
	DMF	39		DMF	37
Cbz-Trp-Phe-NH_2	DMSO	90	Cbz-Trp-Phe-NH_2	DMSO	70
	Methanol	74		Methanol	63
	DMF	20		DMF	50
Cbz-Tyr-Phe-NH_2	DMSO	80	Cbz-Tyr-Phe-NH_2	DMSO	69
	Methanol	49		Methanol	53
	DMF	45		DMF	40
Cbz-Pro-Phe-NH_2	DMSO	0	Cbz-Pro-Phe-NH_2	DMSO	42
	Methanol	0		Methanol	2
	DMF	0		DMF	7

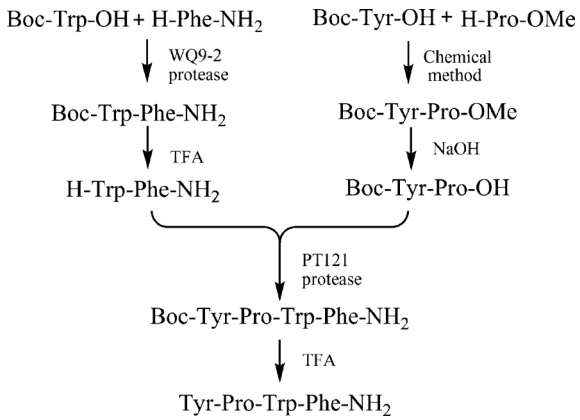

Fig. 1 The strategy of endomorphin-1 synthesis.

Results and discussion

Catalytic specificity of protease WQ9-2 and PT121 in a polar organic–aqueous monophasic system

The catalytic specificity of the two organic solvent-tolerant proteases, which were isolated and developed by us,[14,15] was investigated in 50% (v/v) DMSO, methanol and DMF (Table 1). The synthesis rates in the 50% DMSO system were much faster than that in the buffer system (see the ESI†). Proteases WQ9-2 and PT121 showed a broad and distinct catalytic specificity for carboxylic acid residues with phenylalanine (Phe) on the amine side of the peptide bond. The aromatic residues on the carboxyl side were favorable for protease WQ9-2, while the acidic residue (Asp) was favorable for protease PT121. Protease PT121 showed a moderate catalysis ability in the synthesis of Cbz-Pro-Phe-NH_2, but no Cbz-Pro-Phe-NH_2 was synthesized by protease WQ9-2.

The results also demonstrated that the reaction medium significantly affected the catalysis ability. Higher yields of most tested dipeptides synthesized were observed in 50% DMSO medium, while the yields of dipeptides containing hydrophobic aliphatic residues on the carboxyl component were significantly increased in 50% methanol medium. A moderate catalysis ability in the synthesis of Cbz-Pro-Phe-NH_2 was obtained by protease PT121 in 50% DMSO medium, but a very low activity was observed in methanol and DMF. Thus, the efficient synthesis of peptides could be achieved by using a suitable solvent-stable protease and by modulation of the medium.

Enzymatic synthesis of Boc-Trp-Phe-NH_2 in an organic-aqueous monophasic system

On the basis of the preliminary experiments, Boc-Trp-Phe-NH_2 was efficiently synthesized by solvent-stable protease WQ9-2. High yields of Boc-Trp-Phe-NH_2 in different molar ratios of Phe-NH_2 to Boc-Trp were achieved, as shown in Fig. 2. The best yield was seen at a molar ratio of 1 : 1. This is considered significantly economical without spare substrates.

The effects of different organic solvents and the concentration of organic solvent on the yields of Boc-Trp-Phe-NH_2 are shown in Fig. 3. The product, Boc-Trp-Phe-NH_2, was efficiently synthesized by protease WQ9-2, and precipitated from DMSO, methanol and DMF. The highest yields of 97.1 and 95.4% were obtained in 20% methanol and 30% DMSO, respectively.

To elucidate the productivity of the enzymatic approach in this peptide synthesis, the effect of substrate concentration on the yield of Boc-Trp-Phe-NH_2 in 20% methanol was investigated, as shown in Fig. 4a. More than 90% molar yields of Boc-Trp-Phe-NH_2 with substrate concentrations from 100 to 350 mM were achieved, resulting in a large amount of product crystals (Fig. 4b). The decrease in yield at substrate concentrations above 400 mM was thought to be the result of the enzyme being encapsulated by the large amount of product crystals. The maximum concentration (141 g L^{-1}) of Boc-Trp-Phe-NH_2 was obtained when the substrate concentration reached 350 mM.

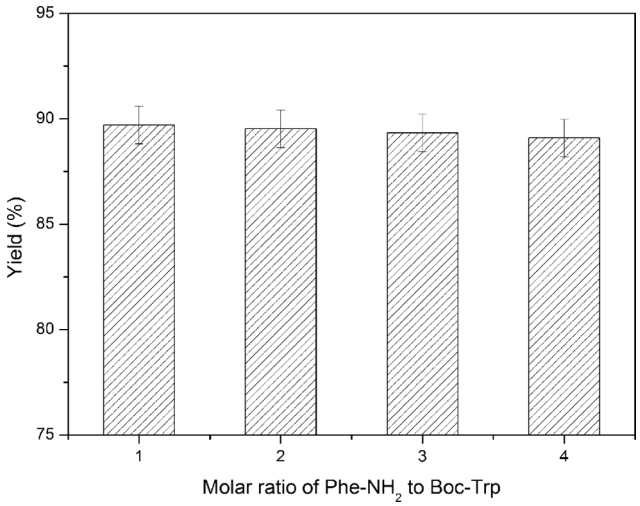

Fig. 2 Effect of the molar ratio of Phe-NH_2 to Boc-Trp (100 mM) on the yield of Boc-Trp-Phe-NH_2. The reactions were catalyzed by protease WQ9-2 (2400 U ml^{-1}) in 50% DMSO medium at 37 °C for 2 h.

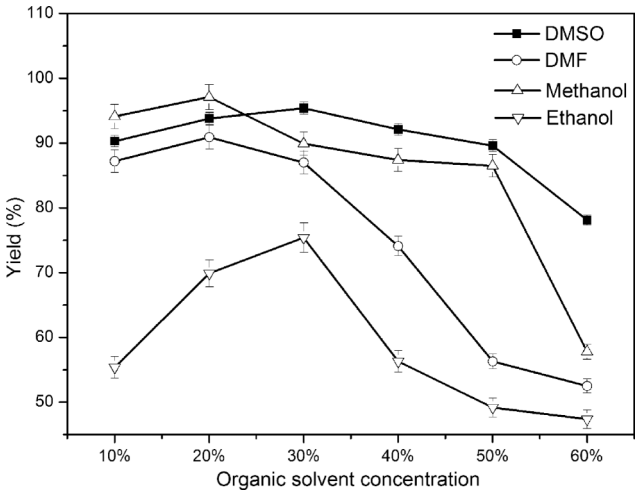

Fig. 3 Effects of concentration of organic solvent on the yields of Boc-Trp-Phe-NH_2. The reactions were performed with 100 mM Boc-Trp and 100 mM Phe-NH_2 using protease WQ9-2 (2400 U ml^{-1}) at 37 °C for 2 h.

The slight decrease in the molar yield of Boc-Trp-Phe-NH_2 with increasing substrate concentration from 100 to 350 mM may be caused by the production of H_2O, resulting in the dilution of the medium.

The efficient preparation of Boc-Trp-Phe-NH_2 in an economical substrate ratio of 1 : 1 was successfully achieved by solvent-stable protease WQ9-2. The product was directly crystallized from the medium and washed with water to generate a 99.6% purity, simplifying the purification process. In contrast, the preparation of Boc-Trp-Phe-NH_2 and analogues using the chemical approach were reported to result in lower yields, and further purification was also needed for each step of the synthesis.[12] The synthesis of Boc-Trp-Phe-NH_2 by immobilized papain was also reported with only a 27.8% yield, and the low yield resulted in a complicated purification.[13]

Chemical synthesis of Boc-Tyr-Pro-OH

Boc-Tyr-Pro-OMe was synthesized by a classical chemical method, the mixed carbonic anhydride method, which is well known for being highly effective at low temperature, resulting in high yields and pure products. Boc-Tyr-Pro-OMe was obtained in a yield of 95.6% and purified simply with washing, based on the significant difference in solubility between the product and substrates. The Boc-Tyr-Pro-OMe was saponified with NaOH to generate Boc-Tyr-Pro-OH (87.4%) for further reaction without purification.

Enzymatic synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2 by protease PT121

Boc-Tyr-Pro-Trp-Phe-NH_2 was synthesized by solvent-stable protease PT121 in an organic–aqueous two-phase medium. The partitioning behavior of the two substrates and the product between the two-phase media was considered to be the key element of synthesis. The yields of Boc-Tyr-Pro-Trp-Phe-NH_2, and the partition coefficients of Boc-Tyr-Pro-OH, Trp-Phe-NH_2 and Boc-Tyr-Pro-Trp-Phe-NH_2 in the presence of various solvents with log *P* values from 0.65 to 4.5 were investigated, as shown in Table 2. The synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2

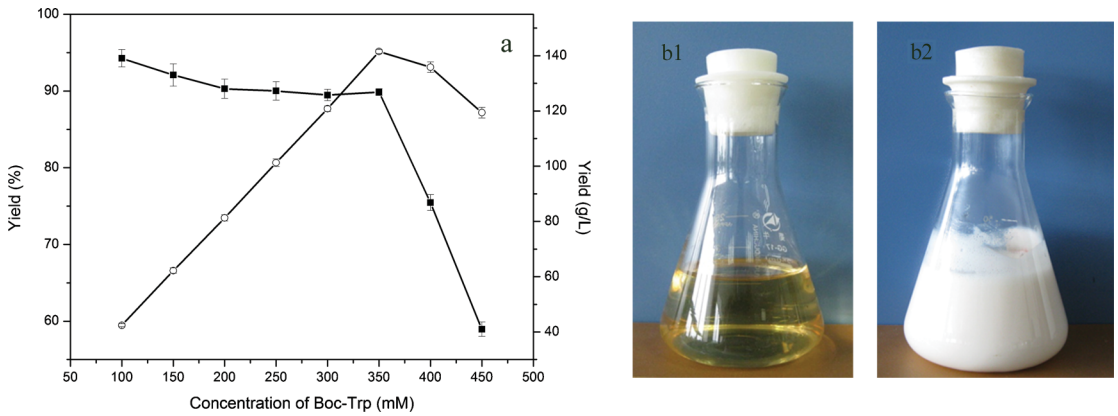

Fig. 4 (a) Effect of substrate concentration on the yield of Boc-Trp-Phe-NH_2. Reactions were performed with a molar ratio of Phe-NH_2 to Boc-Trp of 1 : 1 using protease WQ9-2 (2400 U ml^{-1}) in an initial 20% methanol medium at 37 °C. Symbols: (■) yield (%), (○) yield (g L^{-1}). (b1) Sample catalyzed by inactivated protease WQ9-2. (b2) Product Boc-Trp-Phe-NH_2 synthesized by protease WQ9-2 was crystallized from the 20% methanol medium using protease WQ9-2 with initial substrates of 350 mM Boc-Trp and 350 mM Phe-NH_2.

Table 2 Yield of Boc-Tyr-Pro-Trp-Phe-NH_2, and partition coefficients of Boc-Tyr-Pro-OH, Trp-Phe-$NH_2$and Boc-Tyr-Pro-Trp-Phe-NH_2 in the aqueous–organic biphasic system[a]

Organic solvent	Log *P*	K_{s_1}	K_{s_2}	K_p	Yield
Ethyl acetate	0.68	11.0	1.2	29.5	77.6
n-Butyl acetate	1.7	4.7	0.3	19.7	83.8
n-Butanol	0.8	10.4	12.7	42.8	53.4
Isobutanol	0.65	7.8	6.0	23.3	48.4
Isoamyl alcohol	1.3	8.7	3.9	15.3	47.0
Dichloromethane	1.3	2.5	0.1	5.5	46.3
Chloroform	2.0	2.6	0.1	5.0	40.4
n-Hexane	3.5	0	0	0	0
Cyclohexane	3.2	0	0	0	0
n-Octane	4.5	0	0	0	0

[a] Note: Phase volumetric ratio $\varphi_{aqueous/organic} = 1:2$; K_{s_1}, K_{s_2}, K_p: partition coefficients of Boc-Tyr-Pro-OH, Trp-Phe-NH_2 and Boc-Tyr-Pro-Trp-Phe-NH_2 in the organic and aqueous phase, respectively. The reaction was catalyzed by protease PT121 in the organic–aqueous system of a phase volume ratio ($\varphi_{o/w}$) of 2 : 1 with an initial concentration of 12.5 mM Boc-Tyr-Pro-OH and 50 mM Trp-Phe-NH_2.

was achieved when the product was extracted in the organic phase, which shifted the reaction equilibrium towards the synthesis of the product. The low solubility of the product in the solvent phase was not favorable for the condensation reaction; thus, no product was detected when n-hexane, cyclohexane or n-octane was used as the organic phase. The highest yield of the product was achieved when n-butyl acetate was used as the organic phase, followed by ethyl acetate, due to the partition coefficients of the substrates. Based on the low solubility of Boc-Tyr-Pro-OH in the n-butyl acetate–aqueous biphasic system and the higher boiling point of n-butyl acetate, ethyl acetate was chosen as the best solvent for the synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2 by protease PT121.

The effect of different reaction conditions on the yield of the product were investigated, including the phase volume ratio, molar ratio of Trp-Phe-NH_2 to Boc-Tyr-Pro-OH at different initial concentrations of Boc-Tyr-Pro-OH, rotation speed and temperature. Fig. 5a shows the significant effect of different phase volume ratios in the organic–aqueous two-phase system on the yield of product. The phase volume ratio ($\varphi_{o/w}$) influenced the partitioning behavior of the substrates. The product yield was very low when the phase volume ratio was 1 : 1 due to severe emulsification under vigorous shaking. The yield increased dramatically when the phase volume ratio was increased from 1 : 1 to 3 : 1, and the highest yield was achieved with a phase volume ratio of 3 : 1.

As shown in Fig. 5b, the effect on yields of different molar ratios of Trp-Phe-NH_2 to Boc-Tyr-Pro-OH at different indicated initial concentrations of Boc-Tyr-Pro-OH (10–50 mM) was investigated. When the initial concentration of Boc-Tyr-Pro-OH was 10 mM, the yield of the product increased significantly with increasing molar ratio of Trp-Phe-NH_2 to Boc-Tyr-Pro-OH. Low yields were obtained when the initial concentration of Boc-Tyr-Pro-OH was above 40 mM. The highest yield was achieved at a molar ratio of 3 : 1, with a slightly lower yield at 2 : 1. Considering the cost, the molar ratio of 2 : 1 (Trp-Phe-NH_2/Boc-Tyr-Pro-OH) was selected for the enzymatic synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2. The optimal yield of Boc-Tyr-Pro-Trp-Phe-NH_2 catalyzed by protease PT121 in the organic–aqueous biphasic system was achieved at a rotation of 200 rpm and a temperature of 35 °C (see the ESI†).

The time course of the synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2 under the optimized conditions is shown in Fig. 6. The yield increased with time and reached equilibrium at about 12 h. A molar yield of 84.5% product to substrate Boc-Tyr-Pro-OH was achieved, and the synthesis was considered to be efficient by using the coupling system of reaction–separation in the ethyl acetate–aqueous biphasic medium. However, the synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2 by immobilized papain in a microaqueous acetonitrile medium was reported to result in a molar yield of 35.2%.[13]

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) was obtained by removing the Boc group from Boc-Tyr-Pro-Trp-Phe-NH_2 using 50% (v/v) trifluoroacetic acid (TFA)/dichloromethane (CH_2Cl_2) at room temperature. A high yield of more than 91% was achieved under mild conditions due to the free protection of the side-chain of the Tyr phenolic hydroxy group.

Endomorphin-1 was finally separated by high speed counter-current chromatography (HSCCC) with a purity of 99.8%. The product was confirmed by high resolution mass spectrometry (HRMS) and NMR analysis: *m/z* (ESI) 611.3 $[M + H]^+$ {found (HRMS): *m/z* 611.2994; calc. $[M + H]^+$ for $C_{34}H_{39}N_6O_5$:

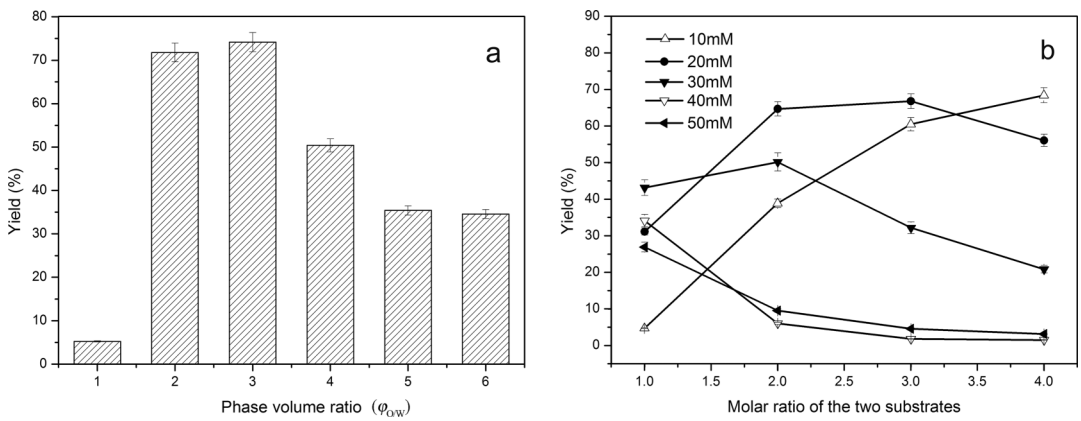

Fig. 5 Effect of reaction parameters on the yield of Boc-Tyr-Pro-Trp-Phe-NH_2 in the ethyl acetate–aqueous system. (a) Effect of phase volume ratio (12.5 mM Boc-Tyr-Pro-OH; 50 mM Trp-Phe-NH_2; 37 °C; 200 rpm). (b) Effect of molar ratio of Trp-Phe-NH_2 to Boc-Tyr-Pro-OH at different indicated initial concentrations of Boc-Tyr-Pro-OH ($\varphi_{o/w} = 3:1$; 37 °C; 200 rpm).

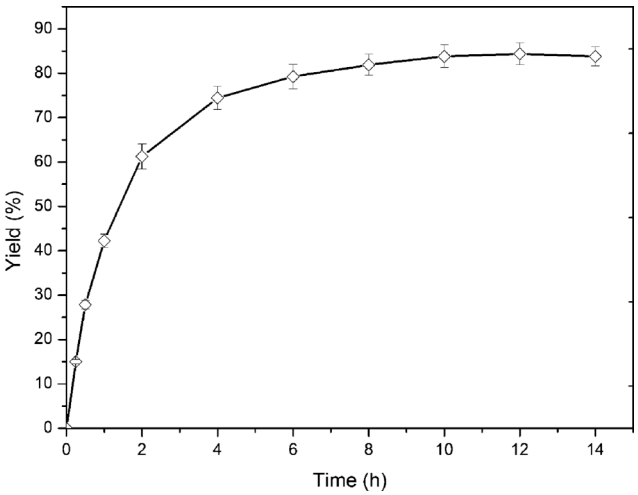

Fig. 6 Time course for the synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2 by protease PT121 in the ethyl acetate–aqueous system with a phase volume ratio ($\varphi_{o/w}$) of 3 : 1 and 20 mM Boc-Tyr-Pro-OH, 40 mM Trp-Phe-NH_2, 35 °C, 200 rpm.

611.2982}. The spectroscopic characterization of Tyr-Pro-Trp-Phe-NH_2 (YPWF) was elucidated with reference to several reports.[16–18] According to the NMR spectra, endomorphin-1 exhibited rotamers (about 2.5/1.0 *trans* to *cis* isomers). ^{1}H-NMR (DMSO, 500 M Hz) δ 10.79 (*trans*) and 10.74 (*cis*) (s,s, 1H, W_{1NH}), 9.43 (*cis*) and 9.35 (*trans*) (s,s, 1H, Y_{OH}), 8.22 (d, J = 8.3 Hz, 1H, W_{NH}), 8.03, 7.85 (dd, J = 8.1 Hz, 2H, Y_{NH_2}), 7.99 (d, J = 7.5 Hz, 1H, F_{NH}), 7.59–7.56 (m, *trans* + *cis*, 1H, W_{2Ar}), 6.90–7.33 (m, 14H, aromatic), 6.70–6.67 (m, *trans* + *cis*, 2H, $Y_{3,5}$), 4.39–4.51 (m, 3H, $P_\alpha W_\alpha F_\alpha$), 4.18 (t, J = 6.0 Hz, 1H, Y_α), 2.77–3.09 (m, 8H, $Y_{\beta1}Y_{\beta2}F_{\beta1}F_{\beta2}W_{\beta1}W_{\beta2}P_{\delta1}P_{\delta2}$), 1.50–1.96 (m, 4H, $P_{\beta1}P_{\beta1}P_{\gamma1}P_{\gamma2}$); ^{13}C-NMR (DMSO, 500 M Hz, *trans* + *cis*) δ 172.6, 172.5, 170.9, 170.8, 170.7, 170.3, 167.0, 156.7, 156.5, 137.7, 136.0, 130.7, 130.3, 129.2, 127.9, 127.3, 127.2, 126.2, 124.6, 124.0, 123.6, 123.2, 120.9, 120.8, 118.2, 115.3, 111.4, 111.2, 109.9, 109.7, 59.6, 59.1, 53.6, 52.5, 46.7, 37.4, 36.3, 35.3, 31.1, 28.8, 27.5, 27.4, 24.4.

Conclusions

Enzymatic catalysis possesses practical beneficial characteristics, such as regio- and stereoselectivity, and could be an important approach in green chemistry. However, enzymatic catalysis is rarely used in industrial chemical synthesis due to the uncoupling of enzyme research with catalysis applications and the non-accessibility of solvent-stable enzymes to chemists. The organic solvent-stable proteases WQ9-2[14] and PT121[15] were isolated from solvent-tolerant bacteria and developed by our group. The efficient synthesis of endomorphin-1 using solvent-stable proteases was successfully achieved. Reaction–separation coupling of Boc-Trp-Phe-NH_2 and Boc-Tyr-Pro-Trp-Phe-NH_2 in the two enzymatic approaches gave a high yield and high productivity. A molar yield of 97.1% in the synthesis of Boc-Trp-Phe-NH_2 (141 g L^{-1}) catalyzed by protease WQ9-2 was achieved in a 20% methanol medium with an economical molar ratio of substrate of 1 : 1. Boc-Tyr-Pro-Trp-Phe-NH_2 with the yield of 84.5% was synthesized by another protease, PT121, in an organic–aqueous biphasic system. The enzymatic synthesis, with high yields in non-aqueous media, simplified the intermediate purification process and was amenable to scale-up.

Experimental

Biological and chemical materials

N-tert-Butoxycarbonyl-L-tyrosine (Boc-Tyr), *N-tert*-Butoxycarbonyl-L-tryptophan (Boc-Trp), L-proline methyl ester (Pro-OMe·HCl), L-phenylaninamide (Phe-NH_2) and trifluoroacetic acid were purchased from GL Biochem (Shanghai, China). Dimethyl sulfoxide (DMSO), methanol and ethanol (all HPLC grade) were purchased from Sinopharm (Shanghai, China). All other chemicals were of analytical grade and purchased from Sunshine (Nanjing, China).

Organic solvent-tolerant proteases WQ9-2 and PT121, from *Bacillus cereus* WQ9-2 and *Pseudomonas aeruginosa* PT121, respectively, were screened and characterized in our previous reports.[14,15,19] The strains of *Bacillus cereus* WQ9-2 and *Pseudomonas aeruginosa* PT121 are currently deposited at the China Center for Type Culture Collection (Wuhan, China) with accession numbers of CCTCCM2010010 and CCTCCM208029, respectively. The genes of the two proteases were also elucidated in patents.[20,21]

Protease production

The organic solvent-tolerant bacteria *Bacillus cereus* WQ9-2 and *Pseudomonas aeruginosa* PT121 were cultured under conditions described previously.[14,19] After incubation, the supernatants were harvested by removing the cells using centrifugation at 10 000 × *g* and 4 °C for 10 min, and used as the crude protease.

Catalytic specificity of protease WQ9-2 and PT121 in a solvent monophasic system

Various carboxyl components of the substrates were used to synthesize the dipeptides with an amine component of Phe-NH_2. The reactions were performed with 100 mM Z-AA and 200 mM Phe-NH_2 in the presence of 50% DMSO, methanol or DMF, respectively. An aliquot of the reaction mixture was withdrawn, and the product and residual substrates analyzed by HPLC.

Enzymatic synthesis of Boc-Trp-Phe-NH_2

Boc-Trp (2.13 g, 350 mM) and Phe-NH_2 (1.15 g, 350 mM) were dissolved in 4 ml methanol, followed by the addition of 16 ml of crude protease WQ9-2 (final concentration of 2400 U ml^{-1}). The reaction mixture was incubated at 37 °C for about 2 h. The product, Boc-Trp-Phe-NH_2, was precipitated as white crystals from the reaction medium with a molar yield of 97.1% and washed with water, resulting in a 99.6% purity. The synthesized Boc-Trp-Phe-NH_2 was confirmed by the mass spectrometry (m/z (ESI) 451.4 $[M + H]^+$). The removal of the Boc group from Boc-Trp-Phe-NH_2 is described below.

Chemical synthesis of Boc-Tyr-Pro-OH

The substrate, Boc-Tyr (0.56 g, 200 mM), was dissolved in 10 ml of dichloromethane, followed by the addition of 0.5 ml *N*-methyl

morpholine with stirring. 0.3 ml isobutylchloro-carbonate was added into the mixture on an ice–salt bath (–15 °C). After 1–2 min activation, Pro-OMe·HCl (0.47 g, 286 mM) was dissolved into the mixture and reacted for 1.5 h. After removal of the solvent *in vacuo*, the residue was dissolved in ethyl acetate, washed with 1 M $NaHCO_3$, 10% (w/v) citric acid and a saturated NaCl solution, then dried with anhydrous $MgSO_4$ and evaporated. Boc-Tyr-Pro-OMe (95.6% yield, 98.3% purity) was obtained as a yellow oil.

Boc-Tyr-Pro-OMe (0.7 g) was then dissolved in 10 ml methanol, followed by 10 ml 1 M NaOH solution. After stirring at room temperature for 10 h, a 20 ml saturated NH_4Cl solution was added. The pH of the solution was then adjusted to 3 with 10% (w/v) citric acid. The mixture was extracted with ethyl acetate and washed with a saturated NaCl solution, dried with anhydrous $MgSO_4$ and evaporated. Boc-Tyr-Pro-OH (87.4% yield, 95.8% purity) was obtained as a white powder. The synthesized Boc-Tyr-Pro-OH was confirmed by mass spectrometry (*m/z* (ESI) 401.1 $[M + Na]^+$).

Enzymatic synthesis of Boc-Tyr-Pro-Trp-Phe-NH_2

Boc-Tyr-Pro-OH (0.042 g, 20 mM) and Trp-Phe-NH_2 (0.056 g, 40 mM) were dissolved in 6 ml water-saturated ethyl acetate. Then, 2 ml of crude protease PT121 (15 000 U ml^{-1}) 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-Trp-Phe-NH_2 was confirmed by mass spectrometry (*m/z* (ESI) 733.9 $[M + Na]^+$).

Removal of the Boc group from Boc-Trp-Phe-NH_2 and Boc-Tyr-Pro-Trp-Phe-NH_2

Removal of the Boc group from Boc-Trp-Phe-NH_2 or Boc-Tyr-Pro-Trp-Phe-NH_2 was based on a typical chemical method. Boc-Trp-Phe-NH_2 or Boc-Tyr-Pro-Trp-Phe-NH_2 (2 mmol) was dissolved in 6 ml dichloromethane, followed by the slow addition of 6 ml trifluoroacetic acid on an ice bath. After the reaction had proceeded at room temperature for 6 h, the solvent was removed under reduced pressure. A mixture (2 : 1) of dichloromethane and methanol was then added and evaporated to remove the residual trifluoroacetic acid. Then, anhydrous diethyl ether (30 ml) was added until the white product precipitated. HPLC and HRMS analyses of the final EM-1 were consistent with a standard sample.

Quantitative analysis of the peptide products

The peptide products were analyzed by HPLC (Dionex P680) using a Kromasil 100-5C18 column (4.6 mm × 250 mm, Kromasil, Sweden) and a UVD170U detector at 280 nm. The mobile phase was solvent A (water) and solvent B (methanol) in the following gradient mode: 0–5 min, 20% B; 5–30 min, 20–100% B; 30–40 min, 100% B. The flow rate of the eluent was 1.0 ml min^{-1} and the oven temperature was 30 °C.

Purification of endomorphin-1

High speed countercurrent chromatography (TAUTO TBE-300B, diameter of tube = 2.6 mm, total volume = 300 ml and a 20 ml sample loop) was applied to separate the final product (Tyr-Pro-Trp-Phe-NH_2). The solvent system for the separation was ethyl acetate–methanol–water (6 : 1 : 6, v/v). The effluent was monitored with a UV-vis detector at 280 nm and the peak fractions were collected separately.

Acknowledgements

Financial support for this research from the National Program on Key Basic Research Project (2009CB724700) and the Key Program of the National Natural Science Foundation of China (20936002) is gratefully acknowledged.

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