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Review

Optimized enzymatic synthesis of C-terminal peptide amides using subtilisin A from *Bacillus licheniformis*

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ABSTRACT

A mild and efficient method for the conversion of C-terminal esters of side-chain protected peptides into an amide function via enzyme-catalysed ammonolysis in organic media with low water content is described. Subtilisin A, the alkaline serine protease from *Bacillus licheniformis*, was used as biocatalyst and ammonium carbamate as source of ammonia. Response surface methodology (RSM) and central composite design were employed to estimate the effects of reaction parameters such as molar ratio of ammonia source to peptide methyl ester (2:1–10:1), composition of the solvent system (Bu^{t}OH/DMF, % v/v, 70:30–95:5) and water concentration (0.2–0.8%) at different temperatures (30–50 °C) for the preparation of Z-Ala-Phe-NH_2 starting from Z-Ala-Phe-OMe. Optimum reaction conditions for maximum amide yield and minimum secondary hydrolysis were determined from cross-section analysis: temperature 30 °C, solvent composition Bu^{t}OH/DMF 82.5:17.5 (v/v) containing 0.2% water (v/v) and molar ratio of ammonia source to peptide methyl ester of 10:1. The maximum yield of Z-Ala-Phe-NH_2 was 87% after 21 h for a quantitative substrate conversion. The method proved to be generally applicable for the synthesis of C-terminal amides of dipeptides with different terminal amino acids and sequence.

Contents

Abbreviations: DP-NH_2, dipeptide amide; DP-OH, dipeptide, free acid; DP-OMe, dipeptide methyl ester; Xaa, any natural amino acid; Bu^{t}OH, *tert*-butanol.

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1. Introduction

Many biologically active peptides contain a C-terminal primary amide function. Known examples are desmopressin, gonadorelin, oxytocin and arginine vasopressin. Amidated peptides can be produced by solid-phase synthesis on benzhydrylamine resins [1]. For the synthesis of peptide amides in solution, which is the method of choice for manufacturing at industrial scale, several strategies may be used. One approach is to start from the C-terminal amino acyl amide, retaining the amide group free along the synthesis, but this is often fraught with severe solubility problems and loss of product in aqueous washings. Another approach is the chemical ammonolysis at elevated pH of C-terminal peptide esters that can be prepared by conventional peptide synthesis, but this entails a considerable risk of racemisation and other side reactions. The use of enzymes to prepare C-terminal peptide amides might prevent all these limitations, due to the mild reaction conditions and the high chemo- and regioselectivity.

Enzymatic synthesis of C-terminal amides of peptides can be carried out by ammonolysis of the C-terminal free acid peptides in a thermodynamically controlled reaction (Scheme 1a) and C-terminal alkyl- or aryl-esters of peptides in a kinetically controlled reaction (Scheme 1b). In the past years, several studies on the enzymatic synthesis of amidated peptides and amino acids using either pathway (a) or (b) were reported. Čeřovskỳ and Kula [2,3] reported the synthesis of C-terminal peptide amides by amidation of the free C-terminal acid group of a peptide substrate using a peptide amidase extracted from orange peel. Despite some optimization of reaction conditions, yields never exceeded 35%, at reaction times ranging from 40 to 144 h [2].

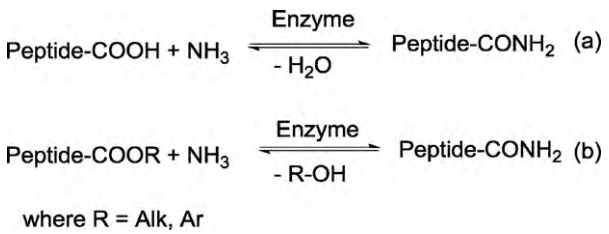
Amidation yields depended strongly on the terminal amino acid, highest yields being obtained for peptides ending with Phe, Met and Leu. The amidase used in these studies showed a strong preference for bulky hydrophobic amino acid residues in the terminal position and had very low activity for hydrophilic or charged amino acid residues. Although feasible, application of this method in solution-phase synthesis of peptide amides is hampered by the low yields, the limited availability of the enzyme and the additional step to deprotect the peptide esters usually produced in the solution-phase synthesis prior to actual amidation.

Synthesis of terminal amides of several N-protected amino acids like Z-Phe-NH_2, Moz-Asp(Bzl)-NH_2, Moz-Glu(Bzl)NH_2, Z-AlaNH_2 and Moz-LeuNH_2 by ammonolysis of peptide methyl or benzyl esters using the industrial alkaline protease Alcalase has been reported by Chen et al. [4]. Reactions were performed in *tert*-butyl alcohol, using ammonium chloride as source of ammonia in the presence of triethylamine at pH 10.6 and higher. Reported yields were between 50 and 70%. Chen also reported the synthesis of the dipeptide Boc-Ala-Phe-NH_2 with a 68% conversion in 12 h. The same reaction was applied for the synthesis of the tripeptide amide Z-Ala-Phe-Leu-NH_2, but the conversion was not mentioned. In all cases, formation of small amounts (3–8%) of the hydrolysis by-product was observed. The reaction was regio- and enantioselective. However, the use of such high pH is unfavourable in the solution-phase synthesis of peptides, since it may result in side reactions depending on the actual sequence.

The use of lipases for the amidation of amino acids [5–7] and other organic acids [8] has also been reported. Du et al. [5], Hacking et al. [6] and Wegman et al. [7] described the synthesis of D-phenylglycine amide using Novozym 435, the immobilized lipase B from *Candida antarctica* (CAL-B), with ammonium carbamate [5] or ammonia [6,7] as source of nucleophilic ammonia. Conversion ranged between 17 and 70%, depending on the length of the alkyl ester chain and the reaction conditions. To the best of our knowledge, the application of lipases for the synthesis of peptide amides, however, has never been reported. In our own studies, we did not detect any significant activity of lipases from various sources, including the free and immobilized CAL-B, for the ammonolysis of C-terminal peptide esters.[1]

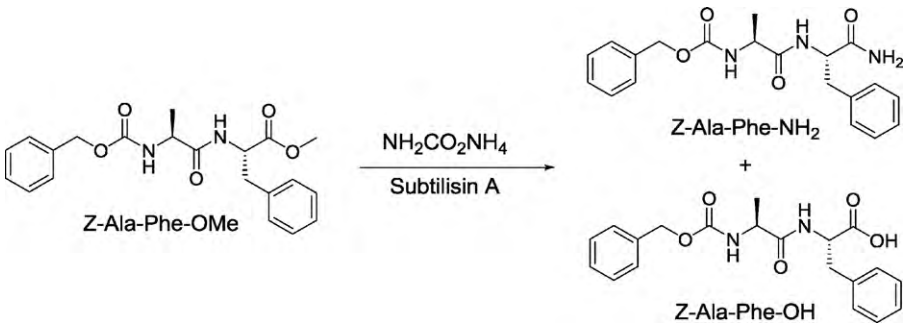
The present work focused on the optimization of the reaction parameters that affect the synthesis of C-terminal peptide amides by ammonolysis of the corresponding methyl esters, catalysed by subtilisin A[2] from *Bacillus licheniformis* in organic solvents with low water content. As model reaction for the optimization, the enzymatic ammonolysis of Z-Ala-Phe-OMe using ammonium carbamate as the source of nucleophilic ammonia was studied (Scheme 2).

In this reaction, besides the main product, Z-Ala-Phe-NH_2, some Z-Ala-Phe-OH was also formed, due to hydrolysis of the peptide methyl ester. Ester hydrolysis is also catalysed by subtilisin, when water is present in the reaction system. Our objectives were to understand the effect of various reaction variables (*i.e.*, substrate molar ratio, composition of the reaction medium, concentration of water in the medium and temperature) on the molar conversion and to obtain the optimum conditions for the highest yield of amide product, while minimising hydrolysis. For this purpose, we applied statistically based experimental design and response surface methodology (RSM), a method that comprises a group of statistical techniques for empirical model building and exploitation [9].



Scheme 1. Pathways for the enzymatic synthesis of peptide amides.

[1] Unpublished data, presented at Biotrans 2009 by C. Boeriu.
[2] Subtilisin A from *B. licheniformis* is the main component of the alkaline protease Alcalase.



Scheme 2. Biocatalytic synthesis of Z-Ala-Phe-NH_2 by subtilisin A in organic media.

2. Materials and methods

2.1. Materials

Subtilisin A from *B. licheniformis* (EC 3.4.21.62) with an activity of 6.3 U/mg was obtained from Sigma–Aldrich (Zwijndrecht, NL). All other chemicals used were of analytical grade.

2.2. Peptide substrates

The C-terminal methyl esters of dipeptides (HPLC purity >98%) and the reference peptides Z-Ala-Phe-NH_2 (HPLC purity 97.4%) and Z-Ala-Phe-OH (HPLC purity 98.4%) were synthesized according to the DioRaSSP procedure [10,11]. The following peptide methyl esters were used as substrates: Z-Ala-Phe-OMe, Z-Val-Phe-OMe, Z-Val-Tyr-OMe, Z-Val-Leu-OMe, Z-Val-Thr-OMe, Z-Val-Ala-OMe, Z-Val-Met-OMe and Z-Val-Lys(Boc)-OMe.[3] All peptides contained less than 0.2% (w/w) of water, as determined by Karl-Fisher titration.

2.3. Enzymatic reactions (experimental design)

Reactions were performed in batch mode in a 24-tubes carrousel (GreenHouse Plus™ Parallel Synthesizer; Radleys Discovery Technologies) with controlled temperature, cooling system and magnetic stirrer. Prior to the reaction, all solvents were dried using molecular sieves 4 Å (10%, w/w) for 24 h, and the water content was determined using the Karl-Fisher method. Z-Ala-Phe-OMe (10 mM) and different molar ratios of ammonium carbamate were added into 5 ml of a solvent mixture composed of different volume ratios of DMF and Bu^{t}OH, containing various amounts of water, and the mixture was thermostated at the temperature *T*. The reaction was initiated by the addition of 10 μl of a 50 mg/ml solution of subtilisin in 0.1 M phosphate buffer pH 7, to achieve a final concentration of enzyme in the reaction mixture of 0.1 mg/ml (corresponding to approximately 0.6 U/ml). The reaction mixture was incubated for 2 h at different temperatures *T*, and then the reaction was quenched by the addition of an equal volume of acetonitrile. Control reactions without enzyme were performed under identical conditions, with the difference that instead of enzyme solution, 10 μl of the 0.1 M phosphate buffer pH 7 was added to the reaction mixture.

Samples were analyzed by RP-HPLC and the identity of the compounds was determined with LC–MS analysis and the use of reference compounds. The molar response factors of the UV-detector for Z-Ala-Phe-OMe, Z-Ala-Phe-NH_2 and Z-Ala-Phe-OH were equal. The results are given as relative percentage yield (molar conversion), defined as shown in Eqs. (1) and (2), and were estimated from peak area integration:

$$\text{Amide yield}\,(\%) = \left[\frac{A_{\text{DP-NH}_2}}{A_{\text{DP-NH}_2} + A_{\text{DP-OH}} + A_{\text{DP-OMe}}}\right] \times 100 \quad (1)$$

$$\text{Free acid peptide yield}\,(\%) = \left(\frac{A_{\text{DP-OH}}}{A_{\text{DP-NH}_2} + A_{\text{DP-OH}} + A_{\text{DP-OMe}}}\right) \times 100 \quad (2)$$

2.4. General procedure for the synthesis of dipeptide amides

10 μl of a solution containing 1 mg subtilisin A in 100 mM sodium phosphate buffer pH 7 was added to 5 ml of an anhydrous mixture of Bu^{t}OH/DMF 82.5:17.5 (v/v), containing 0.05 mmol of the dipeptide methyl ester and 0.5 mmol ammonium carbamate. The mixtures were stirred at 30 °C for 21 h. Reactions were quenched by the addition of an equal volume of acetonitrile, and the samples were analyzed by HPLC and LC–MS. The identity of the compounds was determined with LC–MS analysis and the use of reference compounds, if available.

2.5. Preparative synthesis of Z-Ala-Phe-NH_2

192 mg (0.5 mmol) of Z-Ala-Phe-OMe, 390 mg (5 mmol) ammonium carbamate and 100 μl of a solution containing 10 mg subtilisin A in 100 mM sodium phosphate buffer pH 7 were added to 50 ml of an anhydrous mixture of Bu^{t}OH/DMF 82.5:17.5 (v/v), at 30 °C. The mixture was stirred at 30 °C for 21 h, when the substrate conversion was almost complete (99%, as shown by HPLC). The reaction was stopped by addition of an equal volume of acetonitrile, the mixture was filtered over a G4 glass filter to separate all solids, including enzyme, and the organic phase was concentrated *in vacuo*. A white solid (190 mg, 98.8% yield) was isolated, consisting of Z-Ala-Phe-NH_2 (86.3%) and Z-Ala-Phe-OH (13.7%) as determined by ^{1}H NMR. Characteristics of Z-Ala-Phe-NH_2: R_t (HPLC method) 10.15 min; *m/z* $[M+H]^{1+}$ 370.2; ^{13}C NMR (DMSO-d_6, δ): 172.7, 172.1, 155.7, 137.7, 136.9, 129.2, 128.3, 127.9, 127.8, 127.7, 126.2, 65.4, 53.5, 50.4, 37.4, 17.9; ^{1}H NMR (DMSO-d_6, δ): 7.84 (d, 1H), 7.50–7.10 (m, 8H), 5.02 (dd, 2H), 4.44 (q, 1H), 4.01 (m, 1H), 3.03 (dd, 1H), 2.85 (dd, 1H), 1.13 (d, 3H).

2.6. Analytical methods

2.6.1. High-performance liquid chromatography (HPLC analysis)

The ammonolysis reaction was monitored by HPLC analysis carried out on a HPLC system (Waters) equipped with UV dual wavelength detector and autosampler, using a Vydac 201TP54 (C18, 5 μm, 300 Å, 4.6 mm × 250 mm) reverse-phase (RP) column thermostated at a temperature of 30 °C. The compounds were eluted

[3] The MS, FT-IR and ^{1}H- and ^{13}C NMR characteristics of all peptide substrates and of the reference compounds Z-Ala-Phe-NH_2 and Z-Ala-Phe-OH are given in Supporting information file.

Table 1
Variables used in the experimental design.

Factor	Level		
	−1	0	+1
x_1: molar ratio ammonium carbamate:peptide methyl ester (S2/S1)	2	6	8
x_2: composition of solvent mixture, Bu^{t}OH/DMF (v/v)	70/30	82.5/17.5	95/5
x_3: water concentration (vol%)	0.2	0.5	0.8

using an acetonitrile gradient from 0 to 75% containing 0.1% trifluoroacetic acid, at a flow rate of 2 ml/min. Detection of products and substrates was at 220 and 254 nm.

2.6.2. Liquid chromatography–mass spectrometry (LC–MS analysis)

LC–MS analyses were performed on a Waters Alliance 2695 HPLC, equipped with both UV- (Waters 2996) and MS-detector (Waters ZQ-4000). Conditions for LC analysis were similar to that of HPLC analysis (Section 2.6.1), except the separation column (Atlantis dC18, 3 μm, 2.1 mm × 100 mm) and the flow rate (0.6 ml/min). Mass spectrometric analysis was performed in the positive electrospray mode using a capillary voltage of 3.8 kV, a source temperature of 130 °C. The mass interval was 100–2500 Da; the accuracy of the mass determination is ±0.3.

2.6.3. Fourier transformed infrared spectroscopy (FT-IR) analysis

FT-IR spectra of samples were recorded at ambient temperature using a Varian 1000 FT-IR spectrometer equipped with a MKII Golden GateTM Single Reflection ATR system and a DTGS detector, for the spectral range 650–4000 cm^{-1} at a resolution of 2 cm^{-1}. 128 interferograms were co-added for a high signal to noise ratio.

2.6.4. Nuclear magnetic resonance (NMR) analysis

NMR spectra were recorded on a Bruker Avance III spectrometer operating at 400.17 MHz (^{1}H) and 100.62 MHz (^{13}C). $CDCl_3$ (99.8 at.% D, Aldrich) and DMSO-d_6 (99.9 at.% D, Aldrich) were used as received.

2.7. Optimization of reaction conditions using response surface methodology

2.7.1. Experimental design

Response surface methodology (RSM) was used to describe quantitatively the interactions between reaction parameters and the amide yield. A central composite design was applied, which is especially useful in case of a non-linear response. The response variables studied in this paper consists of amide yield (%) and free acid yield (%). The independent variables and their levels selected for this study are substrate molar ratio, the composition of the solvent system and the water concentration. Table 1 gives the corresponding values of the independent variables at each level. Amidation reactions were carried out according to the arrangement presented in Table 2, for three different temperatures (*i.e.*, 30, 40 and 50 °C), at constant substrate concentration (10 mM of Z-Ala-Phe-OMe) and enzyme to substrate ratio (2.5 wt.%). To avoid bias, experiments were performed in a random order.

2.7.2. Modelling

The response variable is modelled by a quadratic model given as (Eq. (3)):

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (3)$$

Table 2
Central composite design and experimental data for 3-level-3-factor response surface analysis.

Entry	Independent variables			Response variables	
	x_1: S2/S1	x_2: Bu^{t}OH/DMF	x_3: % water	% DP-NH_2	% DP-OH
Experiments at 30 °C					
1	2	82.5/17.5	0.8	21.3	42.4
2	6	82.5/17.5	0.5	56.1	20.8
3	6	70/30	0.8	49.9	26.6
4	2	70/30	0.5	27.9	30.7
5	10	70/30	0.5	63.7	14.4
6	6	95/5	0.8	27.4	19.3
7	10	82.5/17.5	0.8	60.5	22.5
8	6	70/30	0.2	53.1	8.7
9	10	82.5/17.5	0.2	65.6	8.3
10	2	82.5/17.5	0.2	33.8	15.4
11	6	82.5/17.5	0.5	57.7	21.5
12	6	95/5	0.2	28.1	5.6
13	6	82.5/5	0.5	56	19.6
14	2	95/5	0.5	11.7	14.4
15	6	82.5/17.5	0.5	57.8	18.7
16	10	95/5	0.5	12.1	4.4
Experiments at 40 °C					
17	6	82.5/17.5	0.5	53.5	24.5
18	6	95/5	0.2	30.6	5.9
19	6	70/30	0.8	45.2	24.6
20	10	82.5/17.5	0.8	60.9	21.8
21	6	95/5	0.8	30.5	17.2
22	6	82.5/17.5	0.5	57.2	20.4
23	10	95/5	0.5	41.7	10.9
24	10	70/30	0.5	52.9	12.9
25	6	82.5/17.5	0.5	57.4	19.7
26	6	70/30	0.2	42.5	6.2
27	2	70/30	0.5	13.4	24.5
28	2	95/5	0.5	8.4	11.7
29	2	82.5/17.5	0.2	20.9	13.8
30	6	82.5/17.5	0.5	58.4	19.7
31	2	82.5/17.5	0.8	17.2	40.1
32	10	82.5/17.5	0.2	60.5	7.5
Experiments at 50 °C					
33	2	70/30	0.5	14.2	20.6
34	10	82.5/17.5	0.8	58.4	29.9
35	10	70/30	0.5	44.5	13.5
36	6	82.5/17.5	0.5	53.5	23.0
37	10	95/5	0.5	42.8	13.5
38	6	70/30	0.8	39.3	22.8
39	10	82.5/17.5	0.2	55.5	7.9
40	2	82.5/17.5	0.8	17.7	43.3
41	6	82.5/17.5	0.5	53.5	22.1
42	6	82.5/17.5	0.5	54.9	21.6
43	2	82.5/17.5	0.2	24.0	13.6
44	6	95/5	0.2	30.5	6.3
45	6	82.5/17.5	0.5	54.9	21.5
46	2	95/5	0.5	14.0	17.3
47	6	95/5	0.8	36.3	22.9
48	6	70/30	0.2	31.5	5.3

where Y is the response variable, b_0 is the intercept, b_1 to b_{33} the regression coefficients and x_1 to x_3 are the predictors or independent variables. Specific for the experiments in this article: Y is the yield of DP-NH_2 (%) or DP-OH (%), respectively, x_1 is the molar ratio ammonium carbamate:DP-OMe, x_2 is the Bu^{t}OH/DMF (v/v, %) and x_3 is the water concentration in the system (%).

The estimation of the regression coefficients was done by least squares regression. We applied the so-called Mallow's Cp criterion for model selection. This criterion compromises the fit of the model to the response variable with as little as possible regression coefficients in the model to avoid overfitting, that is modelling noise (of experiment) in the model. A log-transformation of the response variable Y is applied, because of the heterogeneity of the residual variance.

Visualisation of the response surface was done using the mathematical software package Matlab. Since in the applied experiments

we have three independent variables and in practice, the response variable can only be visualised as a function of two independent variables, we made cross-sections at different levels of the third independent variable. The figures consist of a combined picture of three cross-section and the three cross-sections separately.

3. Results and discussion

3.1. Model fitting and response surface analysis

One important requirement for achieving successful amidation of (protected) peptide methyl esters by enzymatic ammonolysis is to find an optimum reaction medium with respect to the substrate and the product solubility, and the enzyme stability. Protected peptides as well as their C-terminal methyl esters and amides are generally soluble in polar organic solvents, like DMF, THF, DMSO, acetonitrile, which have a strongly inactivating effect on subtilisin. In previous studies on enzymatic deprotection of C-terminal peptide esters using free and immobilized subtilisin, we have demonstrated that DMF is the most suitable solvent, when used in mixture with a mild co-solvent [12–14]. Therefore, in this study we have used as reaction medium a mixture of anhydrous DMF in Bu^{t}OH to which water (as 0.1 M phosphate buffer pH 7) was added to a total amount below 1%. The concentration of DMF in the solvent mixture was limited to 30% (v/v), to prevent enzyme inactivation.

When a 10 mM solution of the dipeptide methyl ester Z-Ala-Phe-OMe in a mixture of 20% (v/v) DMF in Bu^{t}OH containing 1% of 0.1 M phosphate buffer of pH 7 was treated with an excess of ammonium carbamate (100 mM) and subtilisin A (2.5% weight, corresponding to 0.6 U/ml), a substrate conversion of 64% was obtained after 2 h. The product consisted of a mixture of the dipeptide amide Z-Ala-Phe-NH_2 (44%) and the free acid peptide Z-Ala-Phe-OH (20%) (Fig. 1). In a control reaction without enzyme, neither substrate conversion nor product formation was observed, showing that the reaction measured in the experiment containing subtilisin A was due only to the catalytic effect of the enzyme. Treatment of the dipeptide amide Z-Ala-Phe-NH_2, the main product of the ammonolyis reaction, with subtilisin under similar conditions but without ammonium carbamate did not show any hydrolysis reaction, thus indicating that the free acid peptide Z-Ala-Phe-OH is produced only by the enzyme-catalysed hydrolysis of the C-terminal methyl ester. When amidation of Z-Ala-Phe-OMe with subtilisin and ammonium carbamate was carried out in an anhydrous co-solvent system consisting of 20% DMF and 80% Bu^{t}OH, with no added water, no reaction was observed (data not shown). These results clearly show the essential role of the water content of the reaction medium on the catalytic performance of

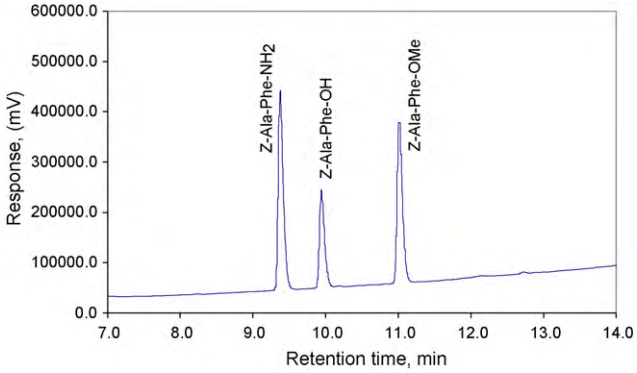


Fig. 1. RP-HPLC chromatogram of the mixture obtained during the enzymatic ammonolysis of Z-Ala-Phe-OMe with ammonium carbamate. Reaction conditions were 10 mM Z-Ala-Phe-OMe, 100 mM ammonium carbamate, solvent system Bu^{t}OH/DMF 80:20 (v/v) with 1% water, 0.1 mg/ml subtilisin A, 40 °C, 2 h, reaction volume 5 ml.

subtilisin in the ammonolysis reaction in polar solvents. When suspended in organic solvents, the enzyme has a rigid conformation, unfavourable for catalysis. Upon addition of a small amount of water to the solvent, the protein main chain can form multiple hydrogen bonds with the surrounding water layer, resulting in an increase of the conformational flexibility of the enzyme and consequently, an increase of the catalytic activity. However, too much water in the reaction system favours the hydrolysis of the substrate and decreases the yield of amidation. Therefore, in our studies, the lower limit of water concentration in the solvent mixture was 0.2% (v/v), to prevent enzyme inactivation, while the highest water content did not exceed 1% (v/v), to avoid undesired substrate hydrolysis.

Another parameter that might influence the yield of C-terminal peptide amide in the kinetically controlled enzymatic ammonolysis of peptide esters is the molar ratio of ammonium carbamate to peptide methyl ester.

To investigate the effect of the reaction parameters on the amide yield, a central composite experimental design was applied. Table 2 shows the independent variables (x_i) and the values of the response variables experimentally obtained. Among the various treatments, the highest amide yield (65.6%) was obtained for entry #9 (S2/S1 10:1, Bu^{t}OH/DMF 82.5:17.5 and water 0.2%, at 30 °C) and the lowest amide yield (8.4%) was for entry # 28 (S2/S1 2:1, Bu^{t}OH/DMF 95:5 and water 0.5%, at 40 °C).

The response variables were modelled to fit the quadratic equation (3), and the results of the regression analysis are given in Table 3. The analysis of variance indicated that the second order

Table 3
Results of estimated parameters of regression analysis of central composite design experiment.

Parameters	T: 30 °C		T: 40 °C		T: 50 °C	
	log(% DP-NH_2)	log(% DP-OH)	log(% DP-NH_2)	log(% DP-OH)	log(% DP-NH_2)	log(% DP-OH)
b_0	−18.34	−13.96	−15.53	−13.81	−14.5	−16.46
b_1	0.725	0	0.543	−0.296	0.3846	−0.1232
b_{11}	−0.0265	0	−0.03144	0	−0.02439	0.00614
b_2	0.523	0.4226	0.437	0.4102	0.4116	0.4445
b_{22}	−0.003253	−0.002681	−0.002735	−0.00267	−0.002504	−0.002694
b_3	0	1.819	0	4.298	−0.46	4.744
b_{33}	0	0	0	−2.388	0.0842	−2.561
b_{12}	−0.00396	−0.001221	0	0.00285	0	0
b_{13}	0	0	0	0	0	0
b_{23}	0	0	0	0	0	0
R^2	86.3	93	97.8	94.9	97	98
se observations[a]	0.213	0.161	0.0911	0.122	0.0847	0.0791

se observations = $\sqrt{}$ (residual variance of the regression model).

[a] se: standard error.

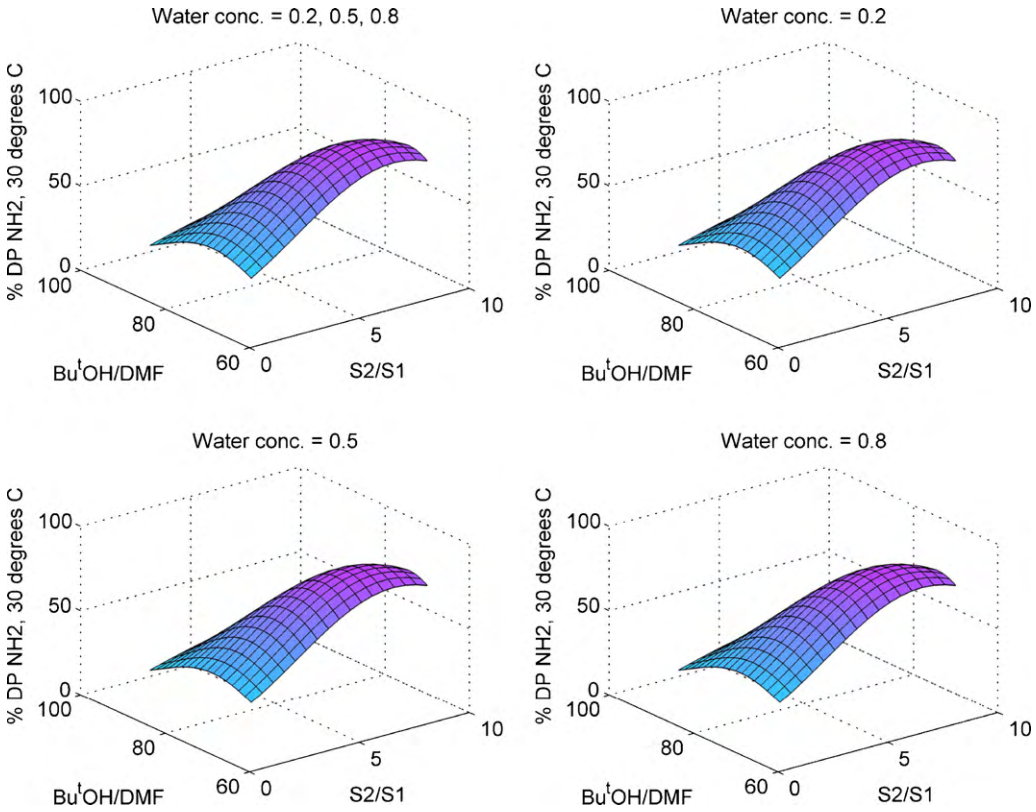


Fig. 2. Response surface plots showing the interaction between solvent composition (Bu^{t}OH/DMF) and substrate molar ratio (S2/S1) at different water concentrations, for amide formation.

polynomial model was statistically significant and adequate to describe the relationship between the response (percent molar conversion) and the significant independent variables, with a satisfactory coefficient of determination (R^2). The results show that the substrate molar ratio (x_1) and solvent mixture composition (x_2) were the most important parameters that showed a statistically significant overall effect on the yield of Z-Ala-Phe-NH_2. Expectedly, the water content (x_3) and solvent mixture composition (x_2) showed a significant effect on the formation of Z-Ala-Phe-OH, the by-product resulting from the hydrolysis of the substrate peptide, Z-Ala-Phe-OMe.

Fig. 2 shows the effect of varying the substrate molar ratio and composition of the reaction medium and their mutual interaction on the amide yield, at different water contents, at 30 °C. As the substrate molar ratio ammonium carbamate:peptide ester increases, the molar yield of the amide Z-Ala-Phe-NH_2 increases, whereas the composition of the solvent system seems to have an optimum around 17.5% DMF and 82.5% Bu^{t}OH. The response surfaces at different water concentrations are identical, suggesting that the variation of water content in the reaction medium from 0.2 to 0.8% has no direct effect on the amide yield. These results are consistent with the zero values of the regression coefficients b_3, b_{33}, b_{13} and b_{23} given in Table 3, which confirm that the water content (x_3) does not have a significant effect on the yield of Z-Ala-Phe-NH_2. Different effects of the substrate molar ratio, the composition of the reaction medium and the water concentration were observed for the formation of Z-Ala-Phe-OH, the product of the ester hydrolysis side reaction (Fig. 3). The formation of Z-Ala-Phe-OH increases as the content of DMF in the solvent mixture increases, and reaches a maximum at a DMF concentration of 17.5%. The increase of substrate molar ratio (x_1) results in a decrease of the yield of Z-Ala-Phe-OH, but the effect is much lower than that observed for the solvent mixture composition x_2. A significant increase of Z-Ala-Phe-OH yield is induced by the increase of the water content (x_3). Also in this case, there was a consensus between the results of the analysis of the response surface and the regression coefficients of the quadratic equation given in Table 3. The analysis of the response surface for Z-Ala-Phe-NH_2 and Z-Ala-Phe-OH at different water concentrations suggests that there is a rapid equilibrium between the enzyme-bound water essential for enzyme activity and the free water in the system, which determines the extent of the ester hydrolysis side reaction. In addition, since the C-terminal free acid peptide coexists with the amide product in the reaction mixture, we may assume that under the conditions of these experiments, the thermodynamically controlled synthesis of peptide amides starting from the C-terminal free acid of peptides, catalysed by subtilisin A, is not favoured.

Similar profiles of the response surface and similar effects of variables x_1, x_2 and x_3 on the yield of Z-Ala-Phe-NH_2 and Z-Ala-Phe-OH respectively were obtained for the models at 40 and 50 °C (not shown).

3.2. Determining optimum conditions

To find the optimal conditions for the subtilisin-mediated ammonolysis of peptide methyl esters that allow maximum amide formation, cross-sections were calculated based on the models given in Table 2, with the following boundary conditions: (1) Z-Ala-Phe-NH_2 yield higher than 50% and (2) Z-Ala-Phe-OH yield lower than 10%.

There are only two cross-sections resulting in an area where the boundary conditions are met (*i.e.*, yield DP-OH < 10% and yield DP-NH_2 >50%) for temperatures equal to 30 and 40 °C, respectively. The other combination of reaction parameters at 50 °C does not have a relevant cross-section. The graphic results of cross-section analysis for 30 °C and 40 °C are given in Fig. 4. The area in pink in

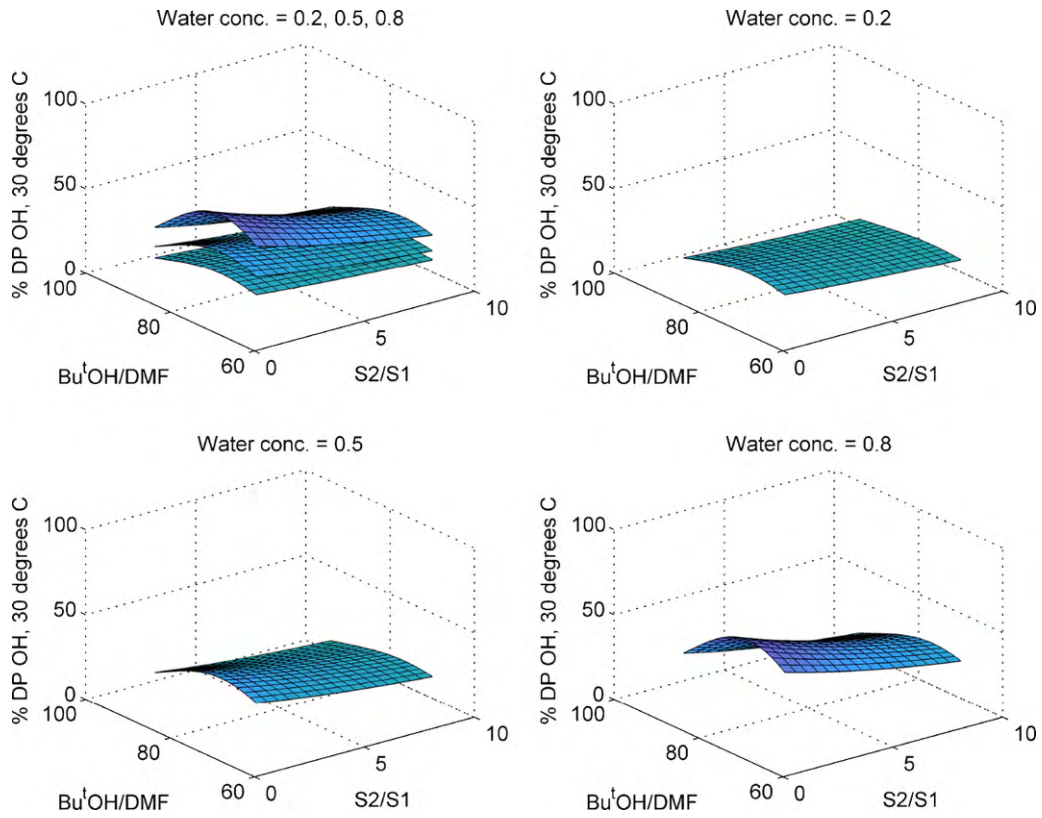


Fig. 3. Response surface plots showing the interaction between solvent composition (Bu^{t}OH/DMF) and substrate molar ratio (S2/S1) at different water concentrations, for by-product formation.

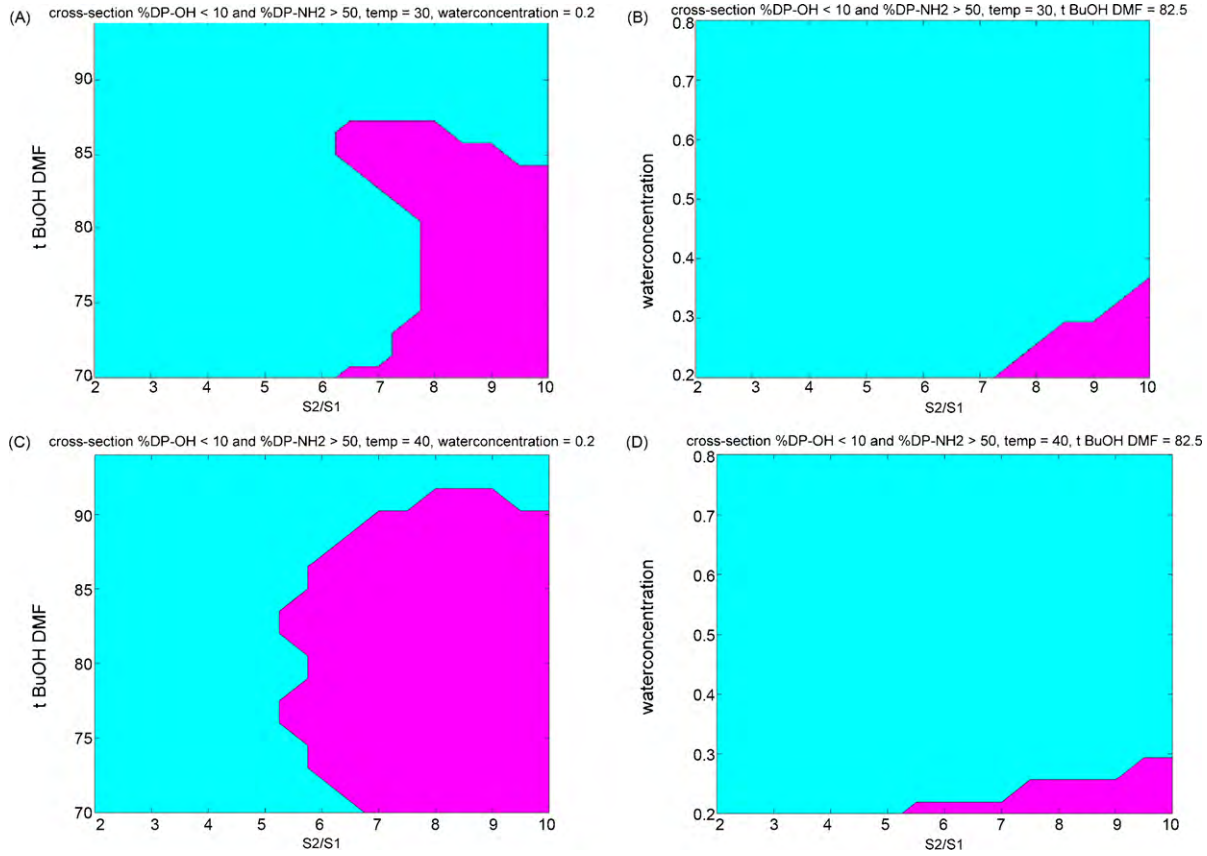


Fig. 4. Visualisation of the by the model predicted area of independent variables for which the boundary conditions % yield DP-NH_2 is larger than 50% and % yield DP-OH is smaller than 10% for 30 °C (A) and 40 °C (B).

Table 4
The interval of the values of independent variables x_i defining the area where boundary conditions % yield DP-NH_2 >50% and % yield DP-OH <10% are completed.

Temperature (°C)	Intervals
30	$8 \le S2/S1 \le 10$ $0.2 \le \% H_2O \le 0.35$ $70/30 \le Bu^tOH/DMF \le 85/15$
40	$6 \le S2/S1 \le 10$ $0.2 \le \% H_2O \le 0.3$ $70/30 \le Bu^tOH/DMF \le 90/10$

Fig. 4 is the area of interest, when the boundary conditions are completed. For each independent variable x_i, the interval describing the three-dimensional space of interest is given in Table 4. At 40 °C, the interval for both variables x_1 (molar ratio S2/S1) and x_2 (medium composition Bu^tOH/DMF) is broader than at 30 °C, while the range in which water concentration (x_3) can be varied is narrower. However, the highest amide yield was obtained at 30 °C (see Table 2), and this might be due to the higher concentration of ammonia in solution at lower temperatures. Based on these results, the following values for the independent variables x_i were selected as optimal: molar ratio S2/S1, 10; solvent composition Bu^tOH/DMF, 82.5:17.5 (v/v); concentration of water in the reaction medium, 0.2 vol%; temperature, 30 °C.

The adequacy of the model developed was examined by performing six additional independent experiments at the selected optimal synthesis conditions, for 2 h reaction time. The molar yield of Z-Ala-Phe-NH_2 and Z-Ala-Phe-OH was 65.4 ± 3.4 and 7.1 ± 0.7%, respectively, which is in agreement with the predicted values.

3.3. Effect of the temperature, reaction time and enzyme concentration on the amide yield

The effect of temperature, enzyme concentration and reaction time on the yield of Z-Ala-Phe-NH_2 was investigated in one-factor-at-a-time analysis. Since results of experimental design showed that both the overall conversion of Z-Ala-Phe-OMe and the amide yield increased with the decrease of temperature (see entries 9, 32, 39 in Table 2), the reaction was carried out at temperatures below 30 °C. Both the total conversion of Z-Ala-Phe-OMe and the amide yield decreased with the decrease of temperature (Fig. 5). The optimal temperature for the amidation of peptide methyl esters with subtilisin is 30–32 °C, close to the conditions when thermal decomposition of ammonium carbamate into ammonia and carbon dioxide is starting, adding to the dissociation of the ammonium

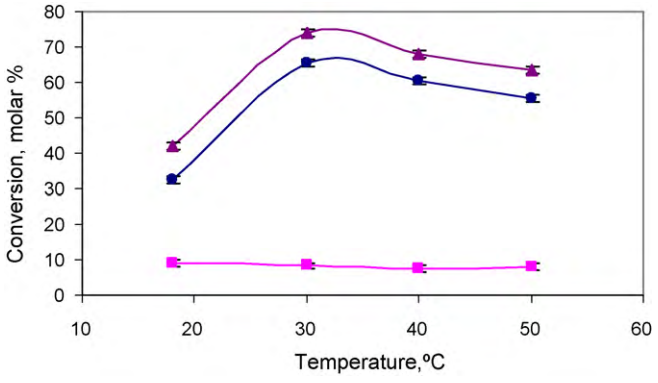


Fig. 5. The effect of temperature on the enzymatic amidation of Z-Ala-Phe-OMe. The curves are: (♦) % molar conversion of Z-Ala-Phe-NH_2, (▲) % molar conversion of Z-Ala-Phe-OMe and (■) % molar conversion of Z-Ala-Phe-OH. Reaction conditions were: 10 mM Z-Ala-Phe-OMe, 100 mM ammonium carbamate, solvent mixture Bu^tOH/DMF 82.5:17.5% (v/v) with 0.2% water, reaction volume 5 ml at an enzyme concentration of 2.7% (w/w of Z-Ala-Phe-OMe) and 2 h.

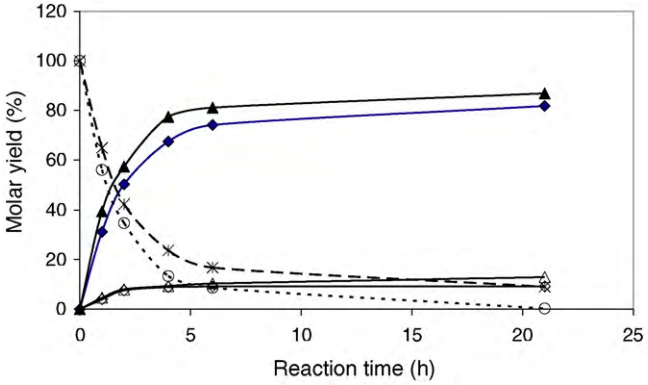


Fig. 6. Time course of the enzymatic synthesis of Z-Ala-Phe-NH_2, at different enzyme concentrations. The curves are: (▲) % molar conversion of Z-Ala-Phe-NH_2, (△) % molar conversion of Z-Ala-Phe-OH, (◯) residual Z-Ala-Phe-OMe (mol%), at an enzyme concentration of 5% (w/w) of Z-Ala-Phe-OMe, and (♦) % molar conversion of Z-Ala-Phe-NH_2, (◊) % molar conversion of Z-Ala-Phe-OH, (×) residual Z-Ala-Phe-OMe, % mol at an enzyme concentration of 2.7% (w/w of Z-Ala-Phe-OMe). Reaction conditions were: 10 mM Z-Ala-Phe-OMe, 100 mM ammonium carbamate, solvent mixture Bu^tOH/DMF 82.5:17.5% (v/v) with 0.2% water, 30 °C, reaction volume 5 ml.

carbamate into carbamic acid and ammonia [15]. No significant temperature effects were observed over the whole range tested (18–50 °C) on the yield of the free acid peptide Z-Ala-Phe-OH by-product. This supports once more the earlier conclusion that the extent of the ester hydrolysis side reaction is controlled by the water content of the medium, which effects the enzyme specific activity. The specific activity of subtilisin for the amidation reaction (18.6 μmol Z-Ala-Phe-NH_2/h mg enzyme) was 12.4-fold higher than that for ester hydrolysis (1.5 μmol Z-Ala-Phe-OH/h mg enzyme), under the optimal conditions for amidation.[4] Fig. 6 shows the time course of the synthesis of Z-Ala-Phe-NH_2 catalysed by subtilisin. By increasing the reaction time from 2 to 21 h, at an enzyme concentration of 0.1 mg/ml (2.7%, w/w, Z-Ala-Phe-OMe) the amide yield increased to 82%, with the formation of 9% of the free acid product (Z-Ala-Phe-OH). The conversion of substrate was 91%. When enzyme concentration was increased to 5.0% (w/w Z-Ala-Phe-OMe), total conversion of substrate Z-Ala-Phe-OMe was obtained. The yield of Z-Ala-Phe-NH_2 after 21 h incubation was 87%, and the product contained some amount of Z-Ala-Phe-OH (Fig. 6).

3.4. Preparative synthesis of Z-Ala-Phe-NH_2

Z-Ala-Phe-NH_2 was enzymatically synthesized at preparative scale. The reaction was carried out at optimum reaction conditions mentioned in Section 3.2, using an enzyme concentration of 0.2 mg/ml. The reaction was stopped after 21 h, when the conversion of substrate Z-Ala-Phe-OMe was above 99% and the amide yield was 84%, as determined from HPLC analysis. The reaction product was isolated as a white powder, in almost quantitative yield. ^1H- and ^{13}C NMR and MS analysis revealed that the major component of the product mixture was Z-Ala-Phe-NH_2. MS analysis and ^1H- and ^{13}C NMR confirmed that Z-Ala-Phe-OH was the sole impurity.

3.5. Application of the method for other peptide substrates

Since the model developed provided optimal conditions for amidation of peptides, we have used the optimal conditions to

[4] The specific activity for amidation and hydrolysis of Z-Ala-Phe-OMe was determined from the initial rate of the formation of Z-Ala-Phe-NH_2 and Z-Ala-Phe-OH, respectively, with (amidation) and without (hydrolysis) ammonium carbamate, at the optimal condition of the amidation reaction.

Table 5
Subtilisin catalysed amidation of Z-Val/Ala-Xaa-OMe dipeptide series in Bu^{t}OH containing 17.5 vol% of DMF and 0.2% water.

Substrate	Composition of the reaction mixture									Ratio amidation/ hydrolysis (mol/mol)
	DP-NH_2			DP-OH			DP-OMe			
	% mol	R_t (min)	m/z $[M+H]^{1+}$	% mol	R_t (min)	m/z $[M+H]^{1+}$	% mol	R_t (min)	m/z $[M+H]^{1+}$	
Z-Val-Phe-OMe	61.0	10.1	398.3	29.1	10.8	399.4	9.9	12.2	413.4	2.1
Z-Val-Tyr-OMe	29.6	8.7	414.5	18.3	9.3	414.3	52.1	10.5	429.3	1.6
Z-Val-Leu-OMe	58.0	9.7	364.3	33.7	10.6	364.4	8.3	12.1	379.5	1.7
Z-Val-Thr-OMe	76.7	7.7	352.4	13.7	8.2	353.2	9.6	9.1	367.3	5.6
Z-Val-Ala-OMe	82.1	7.8	322.3	17.9	8.6	323.4	0.0	9.9	337.4	4.6
Z-Val-Met-OMe	55.7	9.1	382.4	44.3	9.9	383.6	0.0	11.3	396.4	1.3
Z-Val-Lys(Boc)-OMe	10.5	10.4	479.5	6.7	11.0	480.5	82.7	12.2	494.5	1.6
Z-Ala-Phe-OMe	86.8	9.2	370.2	12.9	9.9	371.3	0.3	11.2	385.3	6.7

Conditions: 10 mM of dipeptide; 100 mM of $NH_2CO_2NH_4$; 0.2 mg/ml subtilisin; 5 ml total volume; 21 h; 30 °C.

study the amidation of other dipeptide substrates. The influence of the C-terminal amino acid on the subtilisin-catalysed amidation was investigated using a series of Z-Val/Ala-Xaa-OMe dipeptides, as summarized in Table 5. The results clearly show a high preference of subtilisin A for non-polar amino acid residues in the terminal position, such as Phe, Ala, Met, Thr, Leu, and Tyr. It is remarkable that side-chain protected amino acids in the terminal position, such as Boc-protected Lys, were also amidated. To our best knowledge, this is the first report on the enzymatic synthesis of amides of side-chain protected peptides using subtilisin as catalyst. It appears that the amino acid residue in the P2 position has a strong effect on the specificity and catalytic efficiency of subtilisin. The change of the amino acid residue in the P2 position from Ala (in Z-Ala-Phe-OMe) into Val (in Z-Val-Phe-OMe) results in a decrease of the amide yield from 87 to 61%, and a decrease of the total substrate conversion from 99 to 91%, respectively, as shown in Table 5. Moreover, it seems that the peptide sequence, namely the nature of amino acid residues at both the terminal (P1) and the P2 position has a significant effect on the ratio between the enzymatic amidation and hydrolysis of the peptide methyl esters (see Table 5). At similar substrate conversion, the highest ratio amidation/hydrolysis (6.7) was obtained for peptide Z-Ala-Phe-OMe, and the lowest (1.3) for Z-Val-Met-OMe.

4. Conclusions

In this study, we have developed and optimized a new enzymatic method for the synthesis of free terminal amides of peptides, by ammonolysis of peptide methyl esters using ammonium carbamate and subtilisin A from *B. licheniformis* in polar organic solvents with low water content. The enzyme was very stable and active in a mixture of Bu^{t}OH and DMF 82.5:17.5 (v/v), containing 0.2% water. The optimum conditions for Z-Ala-Phe-NH_2 synthesis were molar ratio ammonium carbamate to Z-Ala-Phe-OMe 10, in Bu^{t}OH/DMF, 82.5:17.5 (v/v) containing 0.2% (v) water, at 30 °C for 21 h with the maximum yield of 87%. The product of the reaction was confirmed as Z-Ala-Phe-NH_2 by LC–MS and NMR. We have also shown that subtilisin A is a suitable catalyst for the synthesis of C-terminal free amides of (protected) peptides with non-polar amino acids at the C-terminus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.03.010.

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