RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

A green route for the synthesis of a bitter-taste dipeptide combining biocatalysis, heterogeneous metal catalysis and magnetic nanoparticles

Vitor A. Ungaro[1#], Cleber W. Liria[1#], Carolina D. Romagna[1#], Natália J. S. Costa[2], Karine Philippot[3], Liane M. Rossi[2], M. Teresa Machini[1*]

[1]*Department of Biochemistry and* [2]*Department of Fundamental Chemistry, Institute of Chemistry, University of São Paulo, PO Box 26077, 05513-970, São Paulo, SP, Brazil.*

[3]*CNRS, LCC (Laboratoire de Chimie de Coordination), 205 route de Narbonne, F-31077, Toulouse cedex 4, France; Université de Toulouse; UPS, INPT; LCC; F-31077 Toulouse, France*

[#] Contributed equally to the present study

[*] Corresponding author (cited as *Machini, MT* or *Miranda, MTM*). Address: Av. Prof. Lineu Prestes, 748 Cidade Universitária, Butantã, 05508-900, São Paulo, Brazil. Tel: +551130913855

E-mail address: mtmachini@iq.usp.br

Abstract

There is increasing demand for green technologies to produce high-solubility and low-toxicity compounds with potential application in the food industry. This study aimed to establish a clean, synthetic route for preparing the bitter-taste dipeptide Ala-Phe, a potential substitute for caffeine as a food additive. Synthesis of Z-Ala-Phe-OMe starting from Z-Ala-OH and HCl.Phe-OMe was catalysed by thermolysin at 50 °C in buffer (Step 1). Z-Ala-Phe-OMe ester hydrolysis to give Z-Ala-Phe-OH at 37 °C in 30% acetonitrile/buffer was catalysed by α-bovine chymotrypsin (αCT), protease with esterase activity (Step 2). Hydrogenation of Z-Ala-Phe to give the desired Ala-Phe was catalysed by C/Pd in methanol (Step 3). Steps 2 and 3 were optimized by using the magnetically recoverable recycling enzyme Fe_3O_4@silica-αCT and the magnetically recoverable metal nanocatalyst Fe_3O_4@silica-Pd, respectively. This inspiring combination of technologies and original results demonstrate the suitability of using enzymes, metal catalyst and magnetic nanoparticles for easy, economical, stereoselective, clean production of an important target compound. Besides, they add to the development of peptide chemistry and catalysis.

Introduction

Peptides found in natural sources or produced in laboratories are structurally and functionally versatile compounds. They are formed by two, a few or dozens of amino-acid residues. They contain only proteinogenic amino acids or, also, pyroglutamic acid, D-amino acids and/or unusual amino acids. In addition, they are: 1) amidated or esterified at the C-terminus; 2) acetylated at the N-terminus; 3) phosphorylated, glycosylated or sulfated in serines, threonines or tyrosines; 4) cyclized through disulfide bonds and 5) lactam-bridged involving amino-acid side-chains or the C- and N-ends[1,2]. They can act as hormones, hormone-releasing factors, neuromodulators, neurotransmitters, toxins, analgesics, antimicrobial agents, flavoring agents, cell-cycle regulators, mitogenic agents, drug carriers, image agents, protease substrates or inhibitors[1,3–12].

The discovery of such diversity has made peptides valuable tools for the biosciences in general, for synthetic organic chemistry, development of novel materials[13] and for the food industry[11,14], creating high demand for methods, procedures and protocols for peptide isolation, purification, separation, identification, quantification and/or synthesis. Consequently, there is continuous need to improve existing methods and need for more productive, more selective and greener methods to prepare peptides in the laboratory.

Increasingly, biocatalysis has been used in the chemistry of amino acids, peptides, proteins, carbohydrates and other biomolecules, due to the enzyme's ability to catalyse chemical reactions chemoselectively, regioselectively and enantioselectively[15]. Indeed, depending on catalytic reactivity and mass transport limits, enzyme-catalysed synthesis can be faster than chemical synthesis. Also, it does not require hazardous substances and may reduce wastes

drastically[16–20]. Therefore, it has been included among the clean, or environmentally friendly, technologies.

In the 1980s, the main advantages of enzyme-catalysed peptide synthesis, including efficiency, high product yields, high selectivity, use of mild reaction conditions and waste minimization, were demonstrated[21–24]. Nevertheless, since then, its main limitation has become apparent: it is mostly applicable to the production of peptides containing up to 5 amino-acid residues[16,25,26]. In addition, it is well known that the technology has disadvantages that make it impractical for reuse and recovery from reaction media, including use of purified proteases and expensive autolytic enzymes with short half-lives in solution. To overcome these limitations and reduce costs, many researchers have immobilized the proteases using a large variety of solid supports[27–30], including nanoparticles[31–33]. Research involving nanoparticles and peptide synthesis can be classified as follows: 1) peptide synthesis followed by interaction of resulting synthetics with nanoparticles to create peptide-nanoparticles for multiple applications[34,35]; 2) use of nanoparticles as supports for solid-phase chemical peptide synthesis[36,37]; 3) peptide-mediated syntheses of nanoparticles, which can be used for a variety of applications[38]; and 4) peptide syntheses catalysed by proteases or esterases immobilized on nanoparticles[28,39,40]. Recently, we have shown that silica-coated superparamagnetic nanoparticles (Fe_3O_4@silica) can be an excellent support for immobilizing bovine α-chymotrypsin (αCT) and that Fe_3O_4@silica-αCT can be reused as a catalyst of peptide ester hydrolysis. In addition, we have demonstrated that Fe_3O_4@silica-αCT can be recovered from reaction media easily and quantitatively by using a magnet[41].

In enzyme-catalysed peptide synthesis and in chemical synthesis, the benzyloxycarbonyl (Z) group (formerly carbobenzoxy (Cbz) group) has been used widely to block amino groups[42].

Z-group removal has been achieved by high-pressure catalytic hydrogenation catalysed by 10% palladium on activated carbon (C/Pd) because this is a clean, relatively fast and, usually, quantitative reaction[43,44]. Nevertheless, C/Pd frequently ignites when it first comes into contact with methanol and to a lesser extent, any flammable organic solvent, thereby representing a significant safety risk. In addition, separation of C/Pd after reaction can be problematic and can result in contamination of the products with metal ions, a serious problem in the pharmaceutical and food industries[45]. Use magnetically responsive nanoparticle catalyst is a safer alternative for catalyst handling, separation and reuse.

Magnetic separation is highly efficient, specific, fast and more advantageous than filtration to isolate a catalyst from simple or complex mixtures[46]. The present study revisited enzyme-mediated peptide synthesis using an approach never used before for preparation of Ala-Phe: a combination of biocatalysis, heterogeneous metal catalysis, superparamagnetic nanoparticles and environmentally friendly solvents. Following is a detailed description of the clean, three-step route developed for this purpose.

Step 1: **Z-Ala-OH + Phe-OMe. HCl → Z-Ala-Phe-OMe + H_2O**
free thermolysin, acetate buffer, pH 6, 50 °C, 6 h

Step 2: **Z-Ala-Phe-OMe + H_2O → Z-Ala-Phe-OH + MeOH**
30% ACN/Tris-HCl buffer, pH 8, 37 °C, 10 min
free bovine αCT (Step 2a, Step 2b) or Fe_3O_4@silica-αCT (Step 2c)

Step 3: **Z-Ala-Phe-OH + H_2 → Ala-Phe + CO_2 + toluene**
1 atm, MeOH, 27 °C, 2 h
C/Pd (Step 3a) or Fe_3O_4@silica-Pd (Step 3b)

Results and Discussion

In this study, the bitter-taste dipeptide Ala-Phe was used as the target compound based on previous studies on the bitterness of amino acids, amino-acid derivatives, dipeptides and tripeptides and because of their high solubility in aqueous solution, low toxicity and potential

application in the food industry. For instance, Kikuchi *et al*.[47] demonstrated that L-Phe, L-Phe-OMe and peptides containing phenylalanine exhibited a bitter taste. In addition, they compared the bitterness of such peptides with that of caffeine, widely used as food additive, and concluded that bitterness increased when L-Phe was the C-terminal amino acid. The dipeptide L-Ala-L-Phe, herein called Ala-Phe, was among those peptides.

Synthesis of the precursor catalysed by free thermolysin (Step 1)

Our search for a cleaner and more cost-effective synthetic route to obtaining Ala-Phe began with synthesis of Z-Ala-Phe-OMe. The choice of thermolysin (metalloprotease produced by the *Bacillus thermoproteolyticus*) as catalyst was based on our previous work on enzymatic synthesis of protected dipeptides[23,48,49] and on earlier reports of Z-Ala-Phe-OMe synthesis in the presence of organic solvents. In fact, Trusek-Holownia[50] made this protected dipeptide in a two-phase ethyl acetate-water system using thermolysin as a catalyst. Later, other researchers synthesized the peptide and its amidated analogue Z-Ala-Phe-NH_2 in various organic or aqueous-organic mixtures using other proteolytic enzymes as catalysts; in such conditions, the product was soluble in the reaction media[51–53].

In the present study, S**tep 1** employed free thermolysin at 50 ^{o}C, because other studies indicated that thermolysin was reasonably stable in the aqueous-organic solutions employed (in the presence of calcium ions, it is significantly less autolytic than other proteases used in enzymatic peptide synthesis[54]) and there are reports on its impressive thermostability (it can catalyse reactions at elevated temperatures)[55]. The experimental conditions used were very similar to those we optimized previously for efficient thermolysin-catalysed synthesis of R^1-Asn-X-OR^2 (where R^1 is an amino-protecting group, X is a hydrophobic amino acid and R^2 is an ester[23,49]). The solvent used was acetate buffer containing calcium acetate (which would keep

thermolysin chemically stable[56] and thus, fully active) and ammonium sulfate (which would help insolubilize Z-Ala-Phe-OMe formed in the reaction medium). Besides being greener than the aqueous-organic mixtures previously used[49-52], this condition allowing product precipitation is one of the experimental approaches used for driving the reactions **R`-CO-NH-R" + $H_2O$ ⇆ R`-COOH + H_2N-R" ⇆ R`-COO^- + ^+H_3N-R"** (where R' and R" represent two peptide chains or two amino acids) toward peptide-bond formation[57,58]. Product precipitation also makes synthesis more economical, as it saves the time, water and electric energy usually required to separate soluble products from reaction media. It also reduces wastes.

Figure 1 shows the time course of peptide-bond formation. In 6 h at 50 °C, it yielded 66% of the Z-Ala-Phe-OMe precipitated in the reaction medium. The solid was simply filtered, washed with distilled water and shown to be homogeneous by LC/ESI-MS (Fig. 2) and salt-free. The yields of the two additional reactions carried out at 25 or 70 °C were 43% and 48%, respectively, indicating that the optimum temperature is about 50 °C. The lower yield observed for the reaction performed at 70 °C may be explained by higher solubility of the dipeptide formed in the reaction medium and/or increased enzyme autolysis and/or enzyme denaturation. Actually, thermolysin denaturing via refolding of the secondary structure was detected by circular dichroism after its incubation for 6 h at 70 °C in the buffer used as solvent for the reactions cited above (data not shown).

In summary, we established environmentally friendly conditions (only buffer) to produce Z-Ala-Phe-OMe efficiently with good yield and high purity.

Ester hydrolysis catalysed by free αCT (Step 2a) or Fe_3O_4@silica-αCT (Step 2b)

Because αCT is a well-known enzyme that catalyses hydrolysis of peptide bonds and esters involving the carboxyl group of aromatic amino acids[59], it was chosen as the biocatalyst

for **step 2 of the synthetic route,** described above. Reactions were not done at temperatures higher than 37 °C because bovine αCT is significantly more autolytic and not as thermostable[60,61] as thermolysin.

At first, a micro-scale reaction starting from Z-Ala-Phe-OMe and using 1 g L^{-1} of free enzyme in reasonably clean conditions (minimum percentage of ACN required for dissolution of Z-Ala-Phe-OMe and buffer, 30%: 70%, v:v) yielded 77% of Z-Ala-Phe-OH in 10 min (**Step 2 a**). The product, isolated with 92% purity as determined by RP-HPLC, had its identity confirmed by LC/ESI-MS (m/z $[M+H^+]$ calculated/found = 371.15/371.2). A subsequent reaction scaled-up 300 times and using an enzyme concentration of 50 g L^{-1} produced 93% of Z-Ala-Phe-OH in 15 min. These results agreed with those of a simultaneous study performed in our laboratories on binding bovine αCT to silica-coated superparamagnetic nanoparticles/examination of the properties of the Fe_3O_4@silica-αCT obtained. That study[41] demonstrated that among other improved properties, the immobilized αCT was able to catalyse peptide ester hydrolysis with the key advantage of being magnetically recoverable and reusable (Primarily due to our interest in establishing the new synthetic route reported here, in that study we also used Z-Ala-Phe-OMe as substrate.).

Then, we attempted to optimize Step 2 by performing two new reactions using significantly less enzyme (25 times less; 0.04 g L^{-1}): one with free αCT (**Step 2b**) and one with Fe_3O_4@silica-αCT (**Step 2c**), both stored in suspension at room temperature, as that would allow comparing performance, advantages and disadvantages. As shown in Figure 3C, in 2 h, the new reaction catalysed by the free enzyme was not as efficient as that catalysed by Fe_3O_4@silica-αCT (55% and 99% of ester hydrolysis, respectively), which was associated with a higher

autolysis of free αCT. Therefore, the new experimental conditions using Fe_3O_4@silica-αCT (**Step 2c**) proved to be even better then **Step 2b** for **Step 2 of the synthetic route**.

Overall, these results are comparable to those obtained previously in enzyme-mediated L-phenylalanine ester hydrolysis[62] and to those reported by Cerovský and coworkers.[63], who used a Z-Ala-Phe-OMe as acyl donor for tripeptide synthesis catalysed by subtilisin Carlsberg. Considering this and the fact that acetonitrile can be recycled and reused easily, we have found reasonably clean, cost-effective conditions under which a magnetically recoverable and reusable enzymatic catalyst efficiently mediates conversion of Z-Ala-Phe-OMe in Z-Ala-Phe.

Z group removal catalysed by C/Pd (Step 3a) or Fe_3O_4@silica-Pd (Step 3b)

Initially, Z-group removal, **Step 3 of the synthetic route** was done by hydrogenation catalysed by C/Pd (**Step 3a**) because this is clean, selective, very productive and, generally, broadly employed in peptide chemistry[43,44] and in organic synthesis[42]. After 2 h, no Z-Ala-Phe, but only the desired product, Ala-Phe, and toluene were detected in the reaction medium. RP-HPLC analysis (Figs. 4a and 4b) confirmed this and gave an excellent yield of 99% (Table 1).

Because our previous study showed that Fe_3O_4@silica-Pd was very efficient as a catalyst of hydrogenation of double[64,65] and triple[66] C-C bonds, this study examined the possibility of replacing C/Pd by Fe_3O_4@silica-Pd[66] in Step 3 (**Step 3b**). If effective, such a step could be even greener than **Step 3a** because such a nanocatalyst does not ignites when it first comes into contact with methanol and is magnetically recoverable from the reaction medium, minimizing time and energy costs compared to C/Pd. In addition, it would be the first proposal of this alternative nanocatalyst for Z-group removal by hydrogenation in peptide synthesis.

At first, the amino acid derivative Z-Phe-OMe was used as a model under the conditions cited above (solvent, nmol of Pd, time) for the reaction catalysed by C/Pd. Figure 5 and Table 1

show that Fe_3O_4@silica-Pd was equally efficient, as, in 2 h, 99% of the esterified amino acid derivative was converted to the unprotected product, H-Phe-OMe. Likewise, hydrogenation of Z-Ala-Phe to give the unprotected dipeptide Ala-Phe was nearly quantitative (Fig. 4c), as, in 2 h, 99% of the desired dipeptide was formed (Table 1). In addition, with the help of a magnet, the Fe_3O_4@silica-Pd mostly was recovered from the reaction media.

In summary, we found a new, efficient, clean, safe condition to perform **Step 3 of the synthetic route** quantitatively based on heterogeneous metal catalysis. The nanocatalyst, never before used for such a purpose, has the potential to be recycled and to be functional for amino acids and peptide chemistry in general.

Experimental

Materials

The amino acid derivatives Z-Ala-OH and HCl-Phe-OMe were from Bachem (California, USA). The thermolysin and α-chymotrypsin were from Sigma Chemical Company (USA) and Biobrás Diagnósticos (Brazil), respectively. The acetonitrile (ACN; Vetec Fine Chemicals Ltd., Brazil) and trifluoroacetic acid (TFA; Merck KGaA, Germany) used to prepare solvents A and B used in RP-HPLC analysis were of spectroscopic grade. The tris(hydroxymethyl)aminomethane (Tris-HCl), calcium acetate, ammonium sulphate, sodium dihydrogen phosphate, hydrochloric acid and methanol (MeOH) were of analytical grade and were purchased from Merck KGaA (Germany). Pd on activated carbon 10 wt% was purchased from Merck Schuchardt OHG (Germany). Tris(dibenzylideneacetone)dipalladium(0) [$Pd_2(dba)_3$] was purchased from Strem Chemicals. (Germany). Other reagents were of analytical grade and commercially available (Aldrich). The synthesis of the catalyst support, Fe_3O_4@silica, was carried out under open-air conditions. The synthesis of the Pd nanocatalyst was carried out under an argon or dinitrogen atmosphere using vacuum-line techniques or a glove box. The hydrogenation reactions were

performed with Fischer-Porter glass reactors connected to a dihydrogen reservoir to maintain pressure.

Synthesis of Z-Ala-Phe-OMe catalysed by free thermolysin

This synthesis was performed using experimental conditions based on our previous studies using thermolysin as a catalyst of peptide bond formation[23,48,49]. Briefly, 0.1 mol L^{-1} of acyl donor (Z-Ala-OH), 0.1 mol L^{-1} of acyl acceptor (Phe-OMe. HCl) and 1 g L^{-1} of enzyme were dissolved in 0.2 mol L^{-1} acetate buffer of pH 6, containing 0.05 mol-L^{-1} of calcium acetate and 200 g L^{-1} of ammonium sulphate reacted for 6 h at 50 °C and 200 rpm. The reaction ended by cooling and separating the insoluble dipeptide formed by centrifugation at 12000 rpm for 6 min in a centrifuge (Model 5415C; Brinkmann Instruments, Inc., Westbury, USA). The supernatant was removed and the insoluble dipeptide washed 3 times with deionized water and dried.

Preparation of superparamagnetic nanoparticle support

The superparamagnetic nanoparticle support comprised preparation of silica-coated magnetite nanoparticles, Fe_3O_4@silica, by a reverse microemulsion and functionalization with amino groups, as previously reported by Rossi *et al*. [67] and Machini *et al*.[41].

Immobilization of αCT on superparamagnetic nanoparticles support

Immobilization of αCT on Fe_3O_4@silica, characterization of an enzyme-nanoparticle hybrid Fe_3O_4@silica-αCT in protein content, amidase activity, chemical and thermal stabilities, efficiency of recovery from the reaction media, reuse potential and ability to catalyse peptide ester hydrolysis all were done as previously described by Machini *et al*.[41].

Ester hydrolysis of Z-Ala-Phe-OMe catalysed by free αCT or Fe_3O_4@silica-αCT

a) Micro-scale reactions

The αCT (1 g L^{-1} or 0.04 g L^{-1}) was incubated with Z-Ala-Phe-OMe (0.02 mol L^{-1}) in a 30% ACN/NaH_2PO_4 buffer (pH 8) for 120 min at 37 ºC and 300 rpm. The reaction was ended by the addition of 6 mL of 50% ACN/0.1% TFA/water. The aliquots were taken in 0, 15, 30, 60 and 120 min for analysis by RP-HPLC, as described below.

b) Milli-scale reaction

Free αCT: The enzyme (50 g L^{-1}) was incubated with Z-Ala-Phe-OMe (0.02 mol L^{-1}) in a 30% ACN/NaH_2PO_4 buffer (pH 8) for 120 min at 37 ºC and 300 rpm. The reaction was ended by the addition of 6 mL of 50% ACN/0.1% TFA/water. The aliquots were taken in 0, 15, 30, 60 and 120 min for analysis by RP-HPLC, as described below. This solution was lyophilized, and the solid was washed with 20 mL ethyl acetate for precipitation of enzymes and salts, which were filtered and washed with 5 mL ethyl acetate. The 2 organic fractions were pooled in a separation funnel to extract water-soluble by-products with 1 mol L^{-1} HCl solution (3 × 5 mL) followed by washing with brine (3 × 5 mL) and removal of the residual water with Na_2SO_4. To finish, the ethyl acetate was evaporated and the product weighed. Most of these steps were monitored by reverse-phase, high-performance liquid chromatography RP-HPLC and/or RP-HPLC coupled to mass spectrometry with electrospray ionization (LC/ESI-MS).

Immobilized αCT: The Fe_3O_4@silica-αCT suspension (0.2 mL) stored at room temperature was placed in a flask, the solid was retained magnetically and the buffer removed. Then, 3.0 mL of a 30% ACN/NaH_2PO_4 buffer (pH 8) and Z-Ala-Phe-OMe (0.04 mol L^{-1}) were added to the reaction flask. The mixture was stirred at 37 °C and 300 rpm. Diluted aliquots were taken at 0, 15, 30, 60 and 120 min of reaction for analysis by RP-HPLC and LC/ESI-MS, as described below.

Preparation of Pd on superparamagnetic nanoparticle support

The catalyst Fe_3O_4@silica-Pd was prepared by the simple approach recently described by Rossi *et al*.[66]. Briefly, Pd NPs were prepared through direct decomposition of the organometallic precursor [$Pd_2(dba)_3$] in the presence of the magnetic support. Initially, 25 mL of a toluene solution of [$Pd_2(dba)_3$] (0.45 mol L^{-1}) was prepared in a Schlenk tube under argon conditions. The solution was transferred under argon with a syringe to a Fischer-Porter reactor containing 1 g of Fe_3O_4@silica-propylamina that had been dried 1 h under vacuum conditions. The mixture was submitted to 3 bars of hydrogen at 25 °C under magnetic stirring for 1 h. After the reaction time, the solid was magnetically recovered, washed with toluene (4 × 6 mL) and dried 1 h under vacuum conditions. The catalyst, denoted as Fe_3O_4@silica-Pd, was stored under open-air conditions. The palladium content of 3.6 wt% was determined by flame atomic absorption spectroscopy (FAAS).

Removal of the Z-group from Z-Phe-OMe and from Z-Ala-Phe-OH catalysed by traditional catalyst or by a superparamagnetic nanoparticle catalyst

The hydrogenation reaction occurred in a Fischer-Porter glass reactor loaded with Z-Phe-OMe or Z-dipeptide (65 mmol L^{-1}), Pd catalyst, (2.3 mg of Pd on activated carbon [C/Pd] or 5.8 mg of Fe_3O_4@silica-Pd, 2 μmmol Pd) and MeOH (1 mL). The reaction mixtures were submitted to 1 bar of H_2 for 2 h at 27 °C and stirring at 700 rpm. Then, catalysts were separated either by centrifugation (C/Pd, 10 min, 13000 rpm) or magnetic separation (Fe_3O_4@silica-Pd, permanent magnet 3500 G). The solution was transferred to another flask for isolation of the deprotected dipeptide, and the catalyst was washed twice with MeOH (1 mL) and once with H_2O (1 mL). After each washing, the catalyst was separated as described above and the supernatant was placed into a single flask. An aliquot of final solution was analyzed by RP-HPLC and LC/MS, as described below.

RP-HPLC and LC/MS analysis

RP-HPLC analyses were done using a Grace C18 Vydac™ column (0.46 cm × 25.00 cm, 5 μmol L^{-1}, 300 Å, USA) connected to a system composed of Constametric 3500 and 3200 pumps from Thermo Separation Products (TSP, USA), an automatic sampler SpectraSYSTEM® AS3000 (TSP, USA), a SpectroMonitor 3100 detector (LDC Analytical, USA) or a Tunable Absorbance Detector 486 (Waters, USA) and a Data Jet integrator (TSP, USA). Separation of compounds (reactants and products) occurred in linear gradients (5–95% B) using 0.1% TFA in water as solvent A and 60% ACN/0.09% TFA in water as solvent B. The flow used was 1 mL/min, and the wavelength (λ) for compound detection was 210 nm.

The LC-MS analyses used the following systems:

1) A Shimadzu liquid chromatographer (Shimadzu Corporation, Kyoto, Japan) composed of 2 LC-10AD pumps, a SDP-10AV detector and a C18 Vydac/Grace column (0.46 cm × 25.00 cm, 5 μmol L^{-1}, 300 Å, USA) coupled to a Micromass Quatro II triple quadrupole mass spectrometer (Altrincham, UK) was used. The capillary voltage was 2kV, the cone voltage was 15kV and MassLynx™ for Windows NT® software was used to analyse mass spectra obtained.

2) A Shimadzu liquid chromatographer (Shimadzu Corporation, Kyoto, Japan) composed of a degasser model DGU-20A3, 2 pumps (model LC-20AD), an injector Rheodyne® 8125, an oven for column (model CTO-20A), a pre-column C18 (5 × 2 mm, 4.6 mm), Shim-pack GVP-ODS and the column C18 (150 × 2 mm, 4.6 μm) Shim-pack VP-ODS coupled to a Bruker Daltonics amaZon® X mass spectrometer (Fahrenheitstrasse, Germany) with ionization source electrospray type, mode ESI positive and an ion-trap analyzer were used. Capillary voltage was 3.5 kV, and the software used for the MS spectra was HyStar 3.2.

In both cases, the results found were comparable to those expected.

Conclusions

Despite using enzyme-peptide synthesis, an older technology, this interdisciplinary study offers original experimental evidence that the combination of biocatalysis, heterogeneous metal catalysis and superparamagnetic nanoparticles is a promising, new tool for peptide chemists. In fact, the study shows that besides being specific and safe, this powerful combination yields green conditions that may be applied to prepare amino acid derivatives, short peptides and other organic compounds derived from them. Fe_3O_4@silica successfully supported both a protease and a metal to furnish the nanocatalysts Fe_3O_4@silica-αCT and Fe_3O_4@silica-Pd and has the potential to be recovered easily from the reaction media by an external magnet and recycled, contributing to development of new, green, synthetic routes of great interest to academia and industry.

Acknowledgments

This work was supported by grants from FAPESP to MTM (2008/11695-1; 2012/09068-4) and fellowships from CNPq and RUSP (to VAU) and FAPESP (to CDR and NJSC). MTM and LMR are members of the NAPCatSinQ-USP.

References

1 M. Matziari, K. Bauer, V. Dive and A. Yiotakis, *J. Org. Chem.*, 2008, **73**, 8591–3.

2 A. Pesic, H. I. Baumann, K. Kleinschmidt, P. Ensle, J. Wiese, R. D. Süssmuth and J. F. Imhoff, *Mar. Drugs*, 2013, **11**, 4834–57.

3 A. Accardo, D. Tesauro, G. Mangiapia, C. Pedone and G. Morelli, *Biopolymers*, 2007, **88**, 115–21.

4 P. Bouziotis, E. Gourni, G. Patsis, D. Psimadas, C. Zikos, M. Fani, S. Xanthopoulos, G. Loudos, M. Paravatou-Petsotas, E. Livaniou, A. D. Varvarigou, I. Pirmettis and M. Papadopoulos, *Bioorg. Med. Chem.*, 2013, **21**, 6699–707.

5 G. Gogoladze, M. Grigolava, B. Vishnepolsky, M. Chubinidze, P. Duroux, M.-P. Lefranc and M. Pirtskhalava, *FEMS Microbiol. Lett.*, 2014, **357**, 63–8.

6 F. Hohla, T. Winder, R. Greil, F. G. Rick, N. L. Block and A. V Schally, *World J. Gastroenterol.*, 2014, **20**, 6102–12.

7 W. E. Kaman, A. G. Hulst, P. T. W. van Alphen, S. Roffel, M. J. van der Schans, T. Merkel, A. van Belkum and F. J. Bikker, *Anal. Chem.*, 2011, **83**, 2511–7.

8 A. Kastin, Ed., *Handbook of Biologically Active Peptides*, Elsevier Inc., 2013.

9 D. H. Kim, I. H. Lee, S. T. Nam, J. Hong, P. Zhang, J. S. Hwang, H. Seok, H. Choi, D. G. Lee, J. Il Kim and H. Kim, *Biochem. Biophys. Res. Commun.*, 2014, **448**, 292–7.

10 E. K. M. Lebbe, S. Peigneur, I. Wijesekara and J. Tytgat, *Mar. Drugs*, 2014, **12**, 2970–3004.

11 V. Nithya, P. S. K. Murthy and P. M. Halami, *J. Appl. Microbiol.*, 2013, **115**, 475–83.

12 G. Sadvakassova, M. C. Dobocan and L. F. Congote, *BMC Res. Notes*, 2009, **2**, 215.

13 B. E. I. Ramakers, J. C. M. van Hest and D. W. P. M. Löwik, *Chem. Soc. Rev.*, 2014, **43**, 2743–56.

14 P. C. Fagundes, H. Ceotto, A. Potter, M. A. Vasconcelos de Paiva Brito, D. Brede, I. F. Nes and M. D. C. D. F. Bastos, *Res. Microbiol.*, 2011, **162**, 1052–9.

15 R. Patel, *Stereoselective Biocatalysis*, CRC Press, 2000.

16 R. J. a C. Beer, B. Zarzycka, M. Mariman, H. I. V Amatdjais-Groenen, M. J. Mulders, P. J. L. M. Quaedflieg, F. L. van Delft, S. B. Nabuurs and F. P. J. T. Rutjes, *Chembiochem*, 2012, **13**, 1319–26.

17 L. Franke, S. Liebscher and F. Bordusa, *J. Pept. Sci.*, 2013.

18 T. Fuchise, H. Sekizaki, H. Kishimura, S. Klomklao, S. Nalinanon, S. Benjakul and B.-S. Chun, *J. Amino Acids*, 2011, **2011**, 912382.

19 N. Ran, L. Zhao, Z. Chen and J. Tao, *Green Chem.*, 2008, **10**, 361.

20 M. T. Reetz, *J. Am. Chem. Soc.*, 2013, **135**, 12480–96.

21 H.-D. Jakubke, P. Kuhl and A. Könnecke, *Angew. Chemie Int. Ed. English*, 1985, **24**, 85–93.

22 A. L. Margolin and A. M. Klibanov, *J. Am. Chem. Soc.*, 1987, **109**, 3802–3804.

23 M. T. M. Miranda, E. Cheng, J. Muradian, W. F. Seidel and M. Tominaga, *Bioorg. Chem.*, 1986, **14**, 182–193.

24 K. Morihara, T. Oka, H. Tsuzuki, Y. Tochino and T. Kanaya, *Biochem. Biophys. Res. Commun.*, 1980, **92**, 396–402.

25 C. W. Liria, C. D. Romagna, N. N. Rodovalho, S. R. Marana and M. T. M. Miranda, *J. Braz. Chem. Soc.*, 2008, **19**, 1574–1581.

26 H. Sekizaki, K. Itoh, A. Shibuya, E. Toyota, M. Kojoma and K. Tanizawa, *Chem. Pharm. Bull. (Tokyo).*, 2008, **56**, 688–691.

27 C. Bernal, A. Illanes and L. Wilson, *Langmuir*, 2014, **30**, 3557–66.

28 A. A. Homaei, R. Sariri, F. Vianello and R. Stevanato, *J. Chem. Biol.*, 2013, **6**, 185–205.

29 D. Li, H. Ding and T. Zhou, 2013.

30 L. Z. Lugo-Morales, P. L. Loziuk, A. K. Corder, J. V. Toups, J. G. Roberts, K. A. McCaffrey and L. A. Sombers, *Anal. Chem.*, 2013, **85**, 8780–6.

31 I. Hegedűs and E. Nagy, *Chem. Eng. Sci.*, 2009, **64**, 1053–1060.

32 C. G. C. M. Netto, H. E. Toma and L. H. Andrade, *J. Mol. Catal. B Enzym.*, 2013, **85-86**, 71–92.

33 J. Kim, J. Lee, H. Bin Na, B. C. Kim, J. K. Youn, J. H. Kwak, K. Moon, E. Lee, J. Kim, J. Park, A. Dohnalkova, H. G. Park, M. B. Gu, H. N. Chang, J. W. Grate and T. Hyeon, *Small*, 2005, **1**, 1203–7.

34 A. Hernández-Gordillo, A. Hernández-Arana, A. Campero and L. I. Vera-Robles, *Langmuir*, 2014, **30**, 4084–93.

35 R. H. Wu, T. P. Nguyen, G. W. Marquart, T. J. Miesen, T. Mau and M. R. Mackiewicz, *Molecules*, 2014, **19**, 6754–75.

36 K. Hojo, A. Hara, H. Kitai, M. Onishi, H. Ichikawa, Y. Fukumori and K. Kawasaki, *Chem. Cent. J.*, 2011, **5**, 49.

37 K. Hojo, H. Ichikawa, M. Onishi, Y. Fukumori and K. Kawasaki, *J. Pept. Sci.*, 2011, **17**, 487–92.

38 Y. Li, Z. Tang, P. N. Prasad, M. R. Knecht and M. T. Swihart, *Nanoscale*, 2014, **6**, 3165–72.

39 H.-Y. Ju, C.-H. Kuo, J.-R. Too, H.-Y. Huang, Y.-K. Twu, C.-M. J. Chang, Y.-C. Liu and C.-J. Shieh, *J. Mol. Catal. B Enzym.*, 2012, **78**, 9–15.

40 B. Xin, S. Si and G. Xing, *Chem. Asian J.*, 2010, **5**, 1389–94.

41 C. W. Liria, V. A. Ungaro, R. M. Fernandes, N. J. S. Costa, S. R. Marana, L. M. Rossi and M. T. Machini, *J. Nanoparticle Res.*, 2014, **16**, 2612.

42 K. Jarowicki and P. Kocienski, *J. Chem. Soc. Perkin Trans. 1*, 2001, 2109–2135.

43 M. Bodanszky, *Peptide chemistry: a practical textbook*, Springer-Verlag Berlin, Heidelberg, 2nd edn., 1993.

44 X. Yang, G. Bai, H. Lin and D. Wang, *Tetrahedron Lett.*, 2014, **55**, 1733–1735.

45 C. E. Garrett and K. Prasad, *Adv. Synth. Catal.*, 2004, 346, 889–900.

46 L. M. Rossi, N. J. S. Costa, F. P. Silva and R. Wojcieszak, *Green Chem.*, 2014, **16**, 2906.

47 N. Ishibashi, K. Sadamori, O. Yamamoto, H. Kanehisa, K. Kouge and E. Kikuchi, 1987, **51**, 3309–3313.

48 M. T. Machini, Universidade de São Paulo, 1985.

49 Ma. T. M. Miranda and M. Tominaga, *Int. J. Pept. Protein Res.*, 2009, **37**, 128–133.

50 A. Trusek-Holownia, *J. Biotechnol.*, 2003, **102**, 153–163.

51 S. R. Morcelle, S. Barberis, N. Priolo, N. O. Caffini and P. Clapés, *J. Mol. Catal. B Enzym.*, 2006, **41**, 117–124.

52 E. Quiroga, N. Priolo, D. Obregón, J. Marchese and S. Barberis, *Biochem. Eng. J.*, 2008, **39**, 115–120.

53 J. Xu, M. Jiang, H. Sun and B. He, *Bioresour. Technol.*, 2010, **101**, 7991–4.

54 G. Fassina, C. Vita, D. Dalzoppo, M. Zamai, M. Zambonin and A. Fontana, *Eur. J. Biochem.*, 1986, **156**, 221–228.

55 V. G. Eijsink, O. R. Veltman, W. Aukema, G. Vriend and G. Venema, *Nat. Struct. Biol.*, 1995, **2**, 374–9.

56 F. W. Dahlquist, J. W. Long and W. L. Bigbee, *Biochemistry*, 1976, **15**, 1103–1111.

57 F. Bordusa, *Chem. Rev.*, 2002, **102**, 4817–4868.

58 A. Machado, C. W. Liria, P. B. Proti, C. Remuzgo, M. Terêsa, D. De Bioquímica, I. De Química, U. D. S. Paulo, A. Prof and L. Prestes, 2004, **27**, 781–789.

59 T. Miyazawa, *Amino Acids*, 1999, **16**, 191–213.

60 A. Kumar and P. Venkatesu, *Chem. Rev.*, 2012, **112**, 4283–307.

61 V. T. Pham, E. Ewing, H. Kaplan, C. Choma and M. A. Hefford, *Biotechnol. Bioeng.*, 2008, **101**, 452–9.

62 E. Flaschel, S. Crelier and A. Renken, *Ann. N. Y. Acad. Sci.*, 1988, **542**, 219–223.

63 J. U. Klein, A. Prykhodzka and V. Cerovský, *J. Pept. Sci.*, 2000, **6**, 541–9.

64 N. J. S. Costa, M. Guerrero, V. Collière, É. Teixeira-Neto, R. Landers, K. Philippot and L. M. Rossi, *ACS Catal.*, 2014, **4**, 1735–1742.

65 M. Guerrero, N. J. S. Costa, L. L. R. Vono, L. M. Rossi, E. V. Gusevskaya and K. Philippot, *J. Mater. Chem. A*, 2013, **1**, 1441.

66 P. M. Uberman, N. J. S. Costa, K. Philippot, R. C. Carmona, A. A. Dos Santos and L. M. Rossi, *Green Chem.*, 2014, **16**, 4566–4574.

67 M. J. Jacinto, P. K. Kiyohara, S. H. Masunaga, R. F. Jardim and L. M. Rossi, *Appl. Catal. A Gen.*, 2008, **338**, 52–57.

Table 1 Catalytic hydrogenation of Z-Ala-Phe or Z-Phe-OMe for 2 h

Z-Compound	Solvent	Catalyst	Reaction yield (%)
Z-Phe-OMe	MeOH	C/Pd	99
Z-Phe-OMe	MeOH	Fe_3O_4@silica-Pd	99
Z-Ala-Phe	MeOH	C/Pd	99
Z-Ala-Phe	MeOH	Fe_3O_4@silica-Pd	99

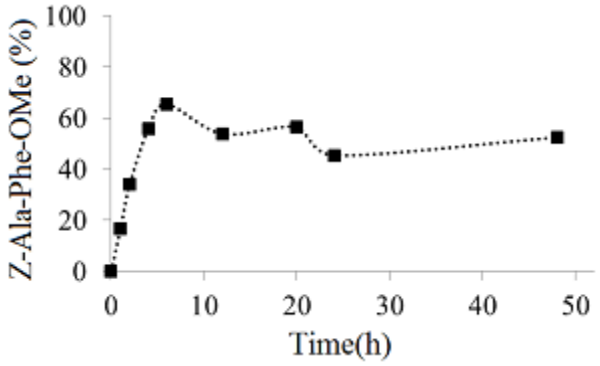

Fig. 1. Reaction course for the synthesis of Z-Ala-Phe-OMe catalyzed by free thermolysin

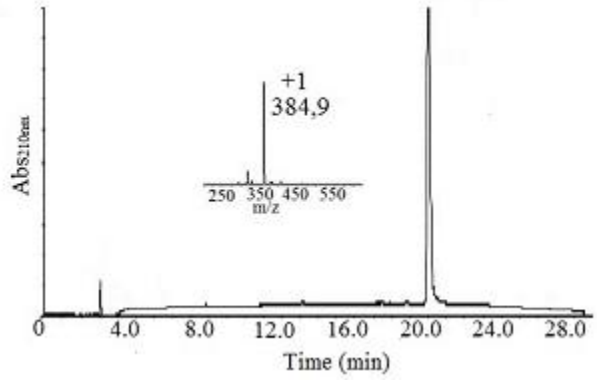

Fig. 2. RP-HPLC monitoring of Z-Ala-Phe-OMe synthesis catalyzed by free thermolysin. **a**: RP-HPLC profile of the reaction medium at 50 °C for 6h; conditions: column Vydac C_{18}, solvent A: 0.1% TFA/H_2O, B: 80% ACN/0.09% TFA/H_2O, flow rate: 1 mL.min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm. **b**: Mass (ESI-MS) spectrum of the component eluted from column at ~21 min; conditions: capillary voltage: 2 kV, cone voltage: 15 kV, ionization mode: ES$^+$. Z-Ala-Phe-OMe, m/z [M+H$^+$] (obtained/calculated): 384.9/385.2

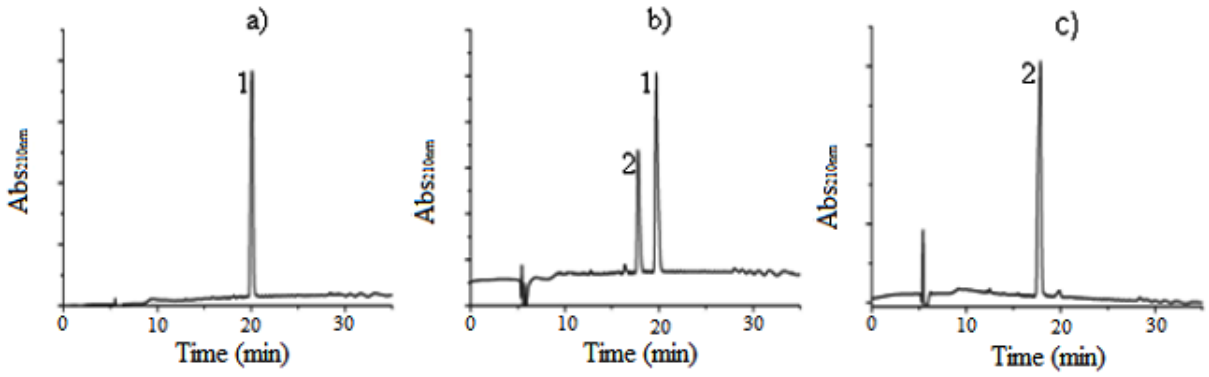

Fig. 3. RP-HPLC monitoring of Z-Ala-Phe-OMe ester hydrolysis catalyzed by αCT to give Z-Ala-Phe. (a) standard Z-Ala-Phe-OMe, (b) reaction catalyzed by free αCT after 2 h, (c) reaction catalyzed by Fe_3O_4@silica-αCT after 2 h. (1) Z-Ala-Phe-OMe and (2) Z-Ala-Phe. RP-HPLC conditions: column Vydac C_{18}, solvent A: 0.1% TFA/H_2O, B: 50% ACN/0.09% TFA/H_2O, flow rate: 1 mL.min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm

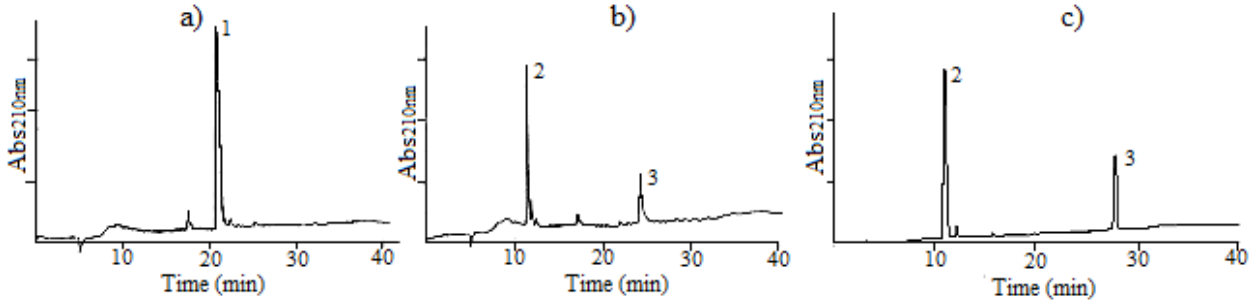

Fig. 4. RP-HPLC monitoring of Z-Ala-Phe hydrogenation catalyzed by C/Pd to give Ala-Phe. (a) 0 h, (b) catalysis by C/Pd after 2 h of reaction, (c) catalysis by Fe_3O_4@silica-Pd after 2 h of reaction. (1) Z-Ala-Phe, (2) Ala-Phe, (3) Toluene. RP-HPLC conditions: column Vydac C_{18}, solvent A: 0.1% TFA/H2O, B: 60% ACN/0.09% TFA/H_2O, flow rate: 1 mL.min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm. MS conditions for the identification of the reaction components: capillary voltage: 4.5 kV, ionization mode: ES^+. Z-Ala-Phe-OH, m/z [M+H+] (obtained/calculated): 371.4/370.4; Ala-Phe, m/z [M+H+] (obtained/calculated): 237.4/236.3

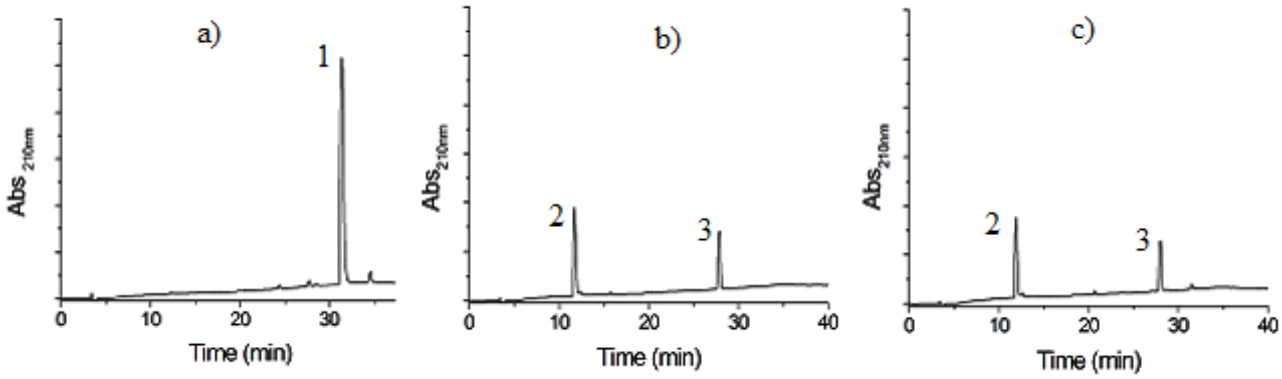

Fig. 5. RP-HPLC monitoring of Z-Phe-OMe hydrogenation to give Phe-OMe. (a) 0 h, (b) catalysis by C/Pd after 2 h of reaction, (c) catalysis by Fe_3O_4@silica-Pd after 2 h of reaction. (1) Z-Phe-OMe, (2) Phe-OMe, (3) Toluene. RP-HPLC conditions: column Vydac C_{18}, solvent A: 0.1% TFA/H_2O, B: 60% ACN/0.09% TFA/H_2O, flow rate: 1 mL. min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm. Mass Spectrometry conditions for the identification of the reaction components: capillary voltage: 4.5 kV, ionization mode: ES^+. Z-Phe-OMe, m/z $[M+H^+]$ (obtained/calculated): 314.4/313.4, Phe-OMe, m/z $[M+H^+]$ (obtained/calculated): 180.3/179.2

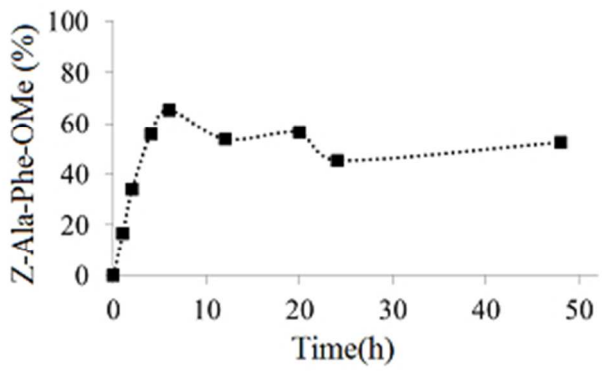

Fig. 1. Reaction course for the synthesis of Z-Ala-Phe-OMe catalyzed by free thermolysin
83x48mm (96 x 96 DPI)

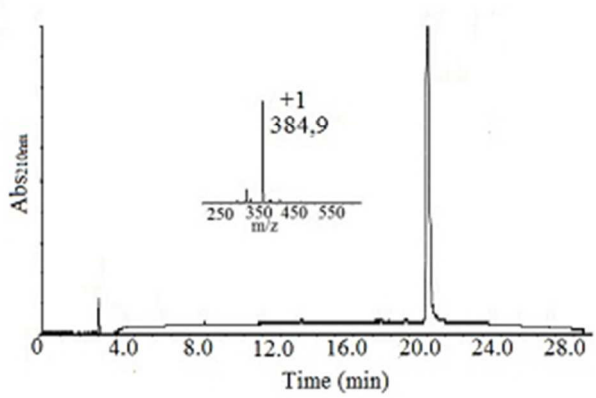

Fig. 2. RP-HPLC monitoring of Z-Ala-Phe-OMe synthesis catalyzed by free thermolysin. a: RP-HPLC profile of the reaction medium at 50 °C for 6 h; conditions: column Vydac C18, solvent A: 0.1% TFA/H_2O, B: 80% ACN/0.09% TFA/H_2O, flow rate: 1 mL min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm. b: Mass (ESI-MS) spectrum of the component eluted from column at ~21 min; conditions: capillary voltage: 2 kV, cone voltage: 15 kV, ionization mode: ES^+. Z-Ala-Phe-OMe, m/z [$M+H^+$] (obtained/calculated): 384.9/385.2
83x54mm (96 x 96 DPI)

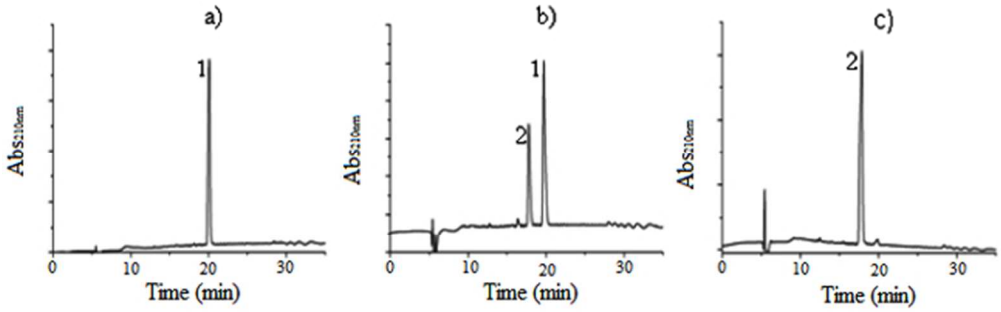

Fig. 3. RP-HPLC monitoring of Z-Ala-Phe-OMe ester hydrolysis catalyzed by αCT to give Z-Ala-Phe. (a) standard Z-Ala-Phe-OMe, (b) reaction catalyzed by free αCT after 2 h, (c) reaction catalyzed by Fe_3O_4@silica-αCT after 2 h. (1) Z-Ala-Phe-OMe and (2) Z-Ala-Phe. RP-HPLC conditions: column Vydac C18, solvent A: 0.1% TFA/H_2O, B: 50% ACN/0.09% TFA/H_2O, flow rate: 1 mL min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm
171x55mm (96 x 96 DPI)

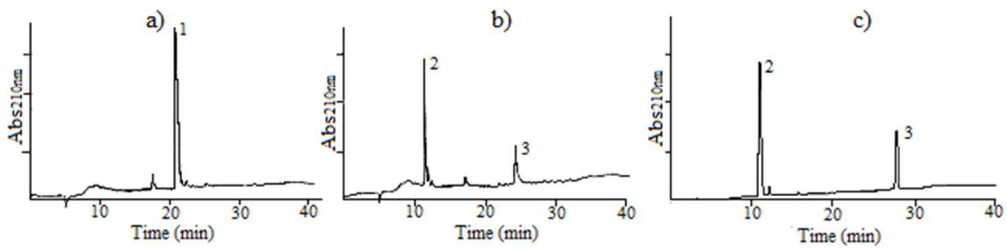

Fig. 4. RP-HPLC monitoring of Z-Ala-Phe hydrogenation catalyzed by C/Pd to give Ala-Phe. (a) 0 h, (b) catalysis by C/Pd after 2 h of reaction, (c) catalysis by Fe_3O_4@silica-Pd after 2 h of reaction. (1) Z-Ala-Phe, (2) Ala-Phe, (3) Toluene. RP-HPLC conditions: column Vydac C18, solvent A: 0.1% TFA/H_2O, B: 60% ACN/0.09% TFA/H_2O, flow rate: 1 mL min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm. MS conditions for the identification of the reaction components: capillary voltage: 4.5 kV, ionization mode: ES^+. Z-Ala-Phe-OH, m/z [$M+H^+$] (obtained/calculated): 371.4/370.4; Ala-Phe, m/z [$M+H^+$] (obtained/calculated): 237.4/236.3
170x43mm (96 x 96 DPI)

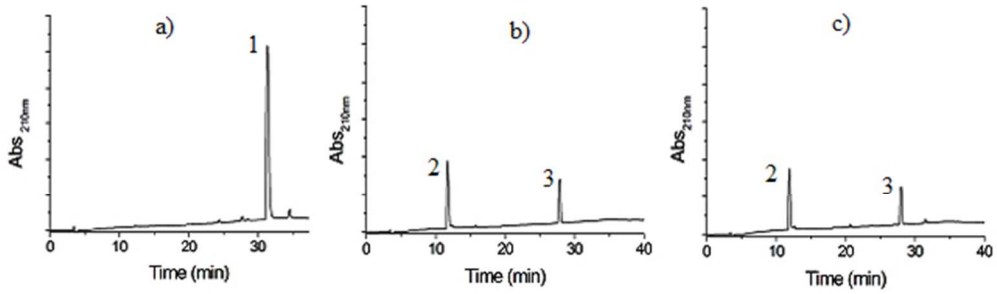

Fig. 5. RP-HPLC monitoring of Z-Phe-OMe hydrogenation to give Phe-OMe. (a) 0 h, (b) catalysis by C/Pd after 2 h of reaction, (c) catalysis by Fe_3O_4@silica-Pd after 2 h of reaction. (1) Z-Phe-OMe, (2) Phe-OMe, (3) Toluene. RP-HPLC conditions: column Vydac C18, solvent A: 0.1% TFA/H_2O, B: 60% ACN/0.09% TFA/H_2O, flow rate: 1 mL min^{-1}, gradient: 5-95% of B in 30 min, λ: 210 nm. MS conditions for the identification of the reaction components:: capillary voltage: 4.5 kV, ionization mode: ES^+. Z-Phe-OMe, m/z $[M+H^+]$ (obtained/calculated): 314.4/313.4, Phe-OMe, m/z $[M+H^+]$ (obtained/calculated): 180.3/179.2

170x51mm (96 x 96 DPI)