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Syntheses of dipeptide alcohols and dipeptide aldehyde precursors catalyzed by plant cysteine peptidases

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ABSTRACT

Two different cysteine peptidases obtained from plant latex (papain from *Carica papaya* and araujiain from *Araujia hortorum*) demonstrated to be good catalysts for the condensation of coded and non-coded Cbz-amino acids and amines such as amino alcohols and amino acetals in acetonitrile containing 1% (v/v) water. Both kinetically and thermodynamically controlled syntheses were proved. Thermodynamic approach was selected since the conversions in product found were similar to those obtained by the kinetic approach; furthermore, a minor number of synthetic steps were needed. For the Cbz-amino acids tested, conversions were higher than 80% at 48–72 h of reaction, except for the Phg derivative, which produced conversions of ca. 40 and 20% for papain and araujiain, respectively. Product yields for the scaled up reactions were similar to the conversions obtained in microscale synthesis. The flexibility of both enzymes for the nucleophile allowed the condensation reaction of Cbz-Ala-OH with an amino diacetal derivative. The resulting dipeptide diacetal derivative can be easily tranformed into a dipeptide aldehyde by acid hydrolysis.

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1. Introduction

Peptides are the focus of intensive studies and their presence in the market is growing in importance, since they can be used as therapeutic drugs, cosmetic ingredients, food additives, and even precursors of soft materials prepared by self assembly [1,2]. The inclusion of non-coded amino acid moieties generates new molecules with novel properties, expanding the application of peptides in the mentioned areas.

Peptide derivatives such as peptide alcohols have interesting applications. Peptaibols are naturally occurring peptide alcohols with antibiotic properties; furthermore, they consist of an interesting group of bioactive peptides of pharmaceutical relevance such as octrotide (i.e., a somatostatin analogue) [3]. Peptide alcohols are also used as ligands or precursors for ligands in asymmetric catalysis, being intermediates for miscellaneous synthesis [4]. They are key intermediates in peptide aldehyde synthesis, which can be used as precursors in the design of protease inhibitors [5]. This feature makes them compounds of utmost importance for the development of drugs with therapeutic application. Peptidases play a key role in the progression of diseases such as hepatitis C (e.g., NS3 protease of hepatitis C virus), Alzheimer's disease (calpains) and AIDS (HIV protease), among many others [6]. Peptide aldehydes yield hemithioacetals and hemiacetals with the catalytic Cys and Ser of cysteine and serine peptidases, mimicking the structure of the reaction transition state. Peptide aldehydes were found to be inhibitors of aspartyl peptidases as well, due to the formation of tetrahedral hydrates of the C-terminus aldehyde function that also mimic the transition state of the substrate during hydrolysis by the enzyme [7]. On the other hand, peptide aldehydes are of great interest for peptide backbone modification or ligation reactions [8].

Non-coded amino acids are of utmost importance since they can be used as drug precursors. Their derivatization with other chemical moieties can lead to diverse building blocks synthesis of interest for commercially significant pharmaceutical products, among others. For example, L-homophenylalanine (L-homoPhe) is the base of several pharmaceutical drugs such as angiotensin converting enzyme inhibitors, β-lactam antibiotics, acetylcholinesterase inhibitors and neutral endopeptidase inhibitors [9]. Phenylglycine (Phg) is also used as a building block for the synthesis of very important pharmaceuticals. D-Phg and its 4-hydroxi derivatives

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are used as key intermediates in the manufacture of penicillin and cephalosporin, whereas L-Phg and its derivatives play also a crucial role in the synthesis of HIV protease inhibitor and taxol, a highly potent anticancer drug [10].

Peptide synthesis can be achieved by different methodologies. Chemical synthesis is probably the most used, since it provides peptides with a precise amino acid sequence in high purity degree. Nevertheless, chemical methods require protection and deprotection steps, as well as the use of toxic reagents. The production of recombinant peptides in culture media is an attractive alternative; however, fermentative production of peptides requires a long and expensive research and development phase, gives low product yield and special methods should be used when non-coded amino acids are included in the peptide sequence [1]. Finally, protease-catalyzed synthesis seems especially suitable for the production of dipeptides; in this sense, the production of peptides having more than two amino acids by this strategy remains a challenging task as it has been widely demonstrated [1,2]. Similarly, peptide alcohols may be synthetized by different strategies, such as coupling with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole (DCC/HOBT) among other related reagents [11], use of aminoacyl chlorides and activated esters as acylating agents, reduction of esters, solid phase synthesis and enzymatic synthesis. Chemical strategies share the same aforementioned drawbacks, though solid phase synthesis is generally claimed as the most appropriate method for the production of this kind of compounds [4,5].

It has been well established that peptidases can catalyze the formation of peptide bonds [12,13]. Moreover, many proteases show substrate promiscuity and they display catalytic activity in reaction conditions far from those they use in physiological conditions [14]. In this context, papain, the cysteine endopeptidase from latex of *Carica papaya*, was found as the most promiscuous from a group of studied proteases [15]. This fact has turn papain into the subject of study of diverse enzymatic syntheses [16,17]. Papain has been extensively used as biocatalyst in different enzymatic amide bond formation reactions, basically peptides and peptide derivatives synthesis [18–25], amino acid esterification with alcohols in a lesser extent [26–29], and other miscellaneous amino acid derivatives using as nucleophiles molecules such as fatty alcohols [30], 4-aminoantipyrine and α-amino-ε-caprolactam [31,32], evidencing its versatility and flexibility. Since papain is the most well-known cysteine peptidase, some modifications were tried in order to improve its catalytic performance. Chemical modifications of papain, such as alkylation of its active site, gave rise to a new catalytic activity of this peptidase, allowing the formation of carbon–carbon bonds by means of cyclisation and dimerization of 6-oxoheptanal [33]. Other chemical modifications with monocarboxylic and dicarboxylic acids led to the acetylation of primary amino groups of the enzyme (ε-amino group of lysine residues), resulting in enhanced catalytic activity for ester formation [34] and higher stability at alkaline pH and thermal stability than unmodified papain [35].

An alternative to chemical/genetic modification of papain is to look for other peptidases with similar biochemical characteristics, but showing a better performance as biocatalysts in the reaction of interest. Araujiain, a crude extract consisting on three papain-like cysteine peptidases, is obtained from the latex of a South American milkweed, *Araujia hortorum* (Asclepiadaceae) [36,37]. Araujiain has demonstrated to be a successful biocatalyst for the synthesis of amide bonds in different aqueous-organic media, and has proved to have a remarkable enzymatic activity deposited onto diverse supports [38–40].

In this opportunity, we report the use of araujiain and papain as biocatalysts for the synthesis of peptide alcohols using Cbz-amino acids (coded and non-coded) as acyl donors and different amino alcohols as nucleophiles. Kinetically and thermodynamically controlled synthesis conditions were tested. Furthermore, the production of a peptide aldehyde by enzymatic catalysis using an amino acetal as nucleophile is also reported in this paper, highlighting the versatility of this kind of peptidases in the condensation reaction between an amino acid and diverse amines.

2. Experimental

2.1. Chemicals

Papain (3.11 units/mg solid, 1 unit hydrolyzes 1.0 mmol of N^{α}-benzoyl-L-arginine ethyl ester chloride [BAEE] per minute at pH 6.2 at 25 °C), molecular sieves (4 Å), ethanolamine and 3-amino-1-propanol were obtained from Fluka. N^{α}-carbobenzyloxy amino acids (Cbz-) such as Cbz-alanine (Cbz-Ala-OH), Cbz-4-amino butyric acid (Cbz-Abu-OH), Cbz-norvaline (Cbz-Nva-OH), Cbz-norleucine (Cbz-Nle-OH), Cbz-phenylglycine (Cbz-Phg-OH), and Cbz-homophenylalanine (Cbz-α-amino-γ-phenylbutyric acid, Cbz-homoPhe-OH) were purchased from Bachem, whereas Cbz-glycine (Cbz-Gly-OH) and Cbz-leucine (Cbz-Leu-OH) were from Sigma. N^{α}-carbobenzyloxy glycine carboxamide methyl ester (Cbz-Gly-OCam) was synthetized according to Baleux et al. [41]. Synthesis of Cbz-Gly-Glyol, Cbz-Ala-Glyol and Cbz-Leu-Glyol used as standards for the enzymatic reactions was performed by activating the amino acid derivatives with *N*-hydroxysuccinimide before coupling with the amino alcohol with DCC by adaptation of methods found in literature for obtaining similar compounds [42,43]. All the amino acid residues used in this work were L-configuration. Thin layer chromatography (TLC) was performed on silica gel sheets 60 F_{254} (Merck). Silica gel grade 62 (60–200 mesh, Grace Davidson) was used for column chromatography. Polyamide-6 (EP-700, particle size <800 μm, mean pore diameter 50–300 nm, specific surface area BET method 8.4 $m^2\,g^{-1}$) and 3,3-dietoxy-propane-1-amine were an Azko (Obernburg, Germany) and Bioglane (Barcelona, Spain) generous contributions, respectively. The rest of the chemicals used in this work were of analytical grade.

2.2. Araujiain extraction and activity determination

Latex from the petiols of unripe fruits (3.35 kg) of *A. hortorum* (Asclepiadaceae) collected in late summer in Arana, La Plata (Buenos Aires Province, Argentina) was gathered in distilled water, containing 5 mM Na_2SO_3 and EDTA as preservatives. The resulting suspension was centrifuged at 9600 × *g* (30 min, 4 °C). The insoluble material (mainly gums) was discarded, and the supernatant containing the soluble proteins (araujiain) was liophylized. To the aim of the work, a general characterization of the proteolytic extract was performed by simple and rapid methods. Protein content was estimated according to Bradford's assay [44], and proteolytic activity was determined using casein as substrate in 0.1 M Tris–HCl buffer pH 8.0 containing 5 mM cysteine at 37 °C. Caseinolytic activity was expressed as an arbitrary enzymatic unit (Ucas), previously defined by Priolo et al. [45].

Protein content and proteolytic activity of commercial papain were estimated using the same methodology in order to perform an appropriate comparison with the proteolytic extract of *A. hortorum* obtained.

2.3. Preparation and characterization of the biocatalysts

Araujiain and papain were deposited onto polyamide in presence of boric acid–sodium borate buffer (0.1 M, pH 8.5) as described before [29]. In order to make a more accurate comparison between both biocatalysts in the synthesis reactions proposed, their activity was calculated using Cbz-Ala *p*-nitrophenyl ester as substrate at pH

8.0 and 37 °C, measuring spectrophotometrically the *p*-nitrophenol produced at 405 nm [14,15]. These assays were made by triplicate, and standard deviation (SD) was calculated. The results were expressed in international units (IU), defined as the micromoles of *p*-nitrophenol released per minute of assay. A calibration curve of *p*-nitrophenol was performed for this purpose.

2.4. Synthesis of peptide alcohols under kinetic control

This approach was tested using Cbz-Gly-OCam (10 mM) as acyl donor and 3-amine-1-propanol (30 mM) as nucleophile in 2 mL of acetonitrile (ACN) containing 1% (v/v) boric acid–sodium borate buffer 0.1 M pH 8.5. ACN was previously dried and stored with molecular sieves (4 Å) under a nitrogen atmosphere. The assay was performed in presence and in absence of the biocatalyst (papain adsorbed onto polyamide, 200 mg) in 4 mL closed vials under a nitrogen atmosphere in an orbital shaker (150 rpm) at 30 °C. Samples (100 μL) were withdrawn at 1, 3, 6, 24, 48 and 72 h. In each case, reactions were stopped by addition of glacial acetic acid (AcH, 20 μL) and the aliquots were analyzed by HPLC (Agilent 1100) in a RP C18 column (Lichrosphere 100 RP-18, 5 μm, 250 mm × 4 mm, Merck). The chromatographic conditions were: solvent A, H_2O 0.1% (v/v) trifluoroacetic acid (TFA); solvent B, ACN/H_2O 80:20 0.1% (v/v) TFA; elution gradient 10% to 70% B in 30 min; flow rate, 1 mL/min; detection at 215 nm. Reactions were made by duplicate, and standard deviation (SD) was calculated.

2.5. Microscale synthesis of peptide alcohols under thermodynamic control

In this case, the stock solution of substrates was prepared as follows. The corresponding amino alcohol (ethanolamine or 3-amino-1-propanol, 100 mM in water) was added dropwise to a solution of acyl donor (0.15 mmoles in water) in order to adjust to a final pH of approximately 7.0. Water was eliminated by lyophilization and the resulting solid was dissolved in a mixture of ACN with 1% (v/v) boric acid–sodium borate buffer 0.1 M pH 8.5. 2 mL of this stock solution (0.02 mmoles of both, acyl donor and nucleophile) were placed in a 4 mL vial and the reaction started by the addition of 200 mg of the biocatalyst tested (papain or araujiain adsorbed onto polyamide). The reaction conditions, sampling, and HPLC analyses were performed as indicated in the previous section. Assays were also made by duplicate and SD was estimated.

Product characterization was accomplished by UPLC–MS and theoretical exact masses were estimated using the ChemCalc online service (http://www.chemcalc.com) and compared with the experimental values obtained (see supplementary material).

2.6. Preparative synthesis of peptide alcohols catalyzed by peptidases

Stocks solutions of Cbz-Gly-OH or Cbz-Ala-OH with the nucleophile (ethanolamine or 3-amino-1-propanol) were prepared as indicated above. Stock solution (10 mL, 0.4 mmoles of acyl donor and nucleophile) was placed in 250 mL flasks and the biocatalyst (papain adsorbed onto polyamide) was added to the mixture. The reaction was kept under nitrogen atmosphere with orbital shaking (120 rpm) at 30 °C for 72 h, and monitored by TLC, with hexane/methanol (80:20). The reaction was stopped by filtering to remove the biocatalyst and the solid washed with ACN. The filtrate was evaporated under vacuum and purified by column chromatography on silica gel using hexane/methanol (80:20) as mobile phase. The eluted fractions were monitored by TLC in the conditions aforementioned. The fractions containing the product were pooled and evaporated under vacuum up to dryness. Each compound was characterized by UPLC–MS as described in Section 2.5, and by ^{1}H NMR and ^{13}C NMR using bidimensional techniques (HSQC) (see supplementary material).

2.7. Condensation reaction between Cbz-Ala-OH and 3,3-dietoxipropane-1-amine catalyzed by peptidases

As described for peptide alcohol synthesis, the stock solution of substrates was prepared by adjusting to pH 7.0 an aqueous solution of Cbz-Ala-OH (0.15 mmoles) with a solution of 3,3-dietoxipropane-1-amine 100 mM. After neutralization, the final solution was lyophilized and the solid residue was dissolved in a mixture of ACN with 1% (v/v) boric acid–sodium borate buffer 0.1 M pH 8.5. Microscale synthesis using either papain or araujiain adsorbed onto polyamide as catalysts was performed as described in Section 2.5; assays were performed by duplicate and SD was calculated. In this particular case, two ways of stopping the reaction were tested: by adding acetic acid as indicated, and placing the reaction vial in an ice-water bath. Product characterization was accomplished by HPLC–MS (Agilent Technologies 1100 Series Quadrupole LC/MS) in the chromatographic conditions already described, with and without adding TFA to the mobile phase. UPLC–MS was also used for characterization in the conditions previously mentioned (see supplementary material).

3. Results and discussion

In this opportunity, araujiain from *A. hortorum* was compared with papain in the condensation reaction between coded and non-coded amino acid derivatives and different functionalized amines after selecting the synthetic approach (kinetic or thermodynamic). Syntheses were performed in ACN with 1% water (v/v), since we already demonstrated that this medium was the most appropriate of a set organic-aqueous miscible systems tested, due to the structural and biological stability shown by papain, the peptidase selected as a model [46].

3.1. Characterization of biocatalysts

Araujiain, the proteolytic extract obtained from latex of the milkweed *A. hortorum*, was characterized in terms of its protein content and specific activity. In this opportunity, the protein content was 67.5 mg/g lyophilized powder (135.1 mg protein/kg fruit) and the specific activity was 5.3 Ucas/mg protein. Determination of the same parameters for commercial papain resulted in a protein content of 0.2 mg protein/g solid, whereas its specific activity was 3.4 Ucas/mg protein.

In order to adjust the enzymatic activity of the biocatalysts for each reaction, and thus to compare their performance in the proposed syntheses, activity was also tested after immobilization using the chromogenic substrate Cbz-Ala *p*-nitrophenyl ester. This substrate is also important since the acyl moiety was used as one of the acyl donors to be proved. The results obtained are shown in Table 1.

It is interesting to point out that before the immobilization step, araujiain specific activity was higher than papain's. As can be observed in Table 1, araujiain seemed to be more affected by adsorption onto polyamide than papain, and thus leading to biocatalysts of almost the same enzymatic activity.

3.2. Selection of synthetic approach. Kinetically-controlled synthesis using an -OCam derivative

Enzymatic peptide synthesis can be achieved by two different approaches, thermodynamic controlled synthesis or kinetic controlled synthesis. Briefly, in the thermodynamic approach, the acyl donor (an N-terminally protected amino acid, in this case) reacts

Table 1
International units (IU) calculated for each immobilized biocatalyst using Cbz-Ala *p*-nitrophenyl ester as substrate.

Biocatalyst	Activity (IU/mg immobilized preparation)	Specific activity (IU/mg immobilized preparation)
Araujiain adsorbed onto polyamide	0.021 ± 0.005	0.99 ± 0.01
Papain adsorbed onto polyamide	0.023 ± 0.002	0.81 ± 0.04

with an acceptor (nucleophile), resulting in the formation of an amide bond, producing one molecule of water. Thermodynamic controlled synthesis is slow and the thermodynamic equilibrium must be shifted towards the synthetic direction by means of product precipitation, water withdrawal, or organic solvents addition. In contrast, the kinetic approach involves a C-terminally activated acyl donor that reacts with the nucleophile to give the product in high yields in generally shorter reaction time than the thermodynamic approach. Bearing this in mind, synthesis under kinetic control is often preferred over the thermodynamically controlled reaction [12,47]. However, thermodynamic controlled synthesis is methodologically straightforward since no acyl donor ester is needed, therefore the free carboxylic acid can be used [12].

For the synthesis of peptide alcohols we first tried the kinetic approach by using Cbz-Gly-OCam as acyl donor and 3-amino-1-propanol as nucleophile. The reason for choosing an -OCam derivative as acyl donor was based on previous reports, which indicated that they gave better results than other esters [48]. In this sense, -OCam derivatives were profusely employed as acyl donors for enzymatic peptide synthesis [23,49] due to its great effectiveness in acylating peptidases active sites [50].

For this first assay, papain was the enzyme tested, whereas a blank reaction with no biocatalyst was also carried out in the same conditions. As can be seen in Fig. 1, papain produced a conversion of almost 40% in the desired product was reached after 1 h of reaction. At 24 h, the conversion was more than 95%, whereas the hydrolysis by-product Cbz-Gly-OH generated was less than 5%. These results would indicate that the Cam esters are very appropriate for this kind of condensation reactions catalyzed by proteases.

However, when the same reaction was performed without biocatalyst, a considerable amount of Cbz-Gly-β-Alaol was also obtained: a conversion of 90% was produced after 24 h. The difference in the ratio of product obtained between catalyzed and non-catalyzed condensation decreased with the increase in the reaction time: after 1 h this difference was approximately 50% (i.e., the amount of Cbz-Gly-β-Alaol produced in presence of papain was twice than in absence of enzyme), whereas at 6 h this difference diminished up to 30%, and in 24 h, the difference was only 10% (Fig. 1). These results indicate that the acyl donor was highly activated. Such activation makes that the -OCam derivatives are able to produce the desired condensation reaction in reasonable good yields without any catalyst addition, a fact that, up to the authors' knowledge, have never been reported before.

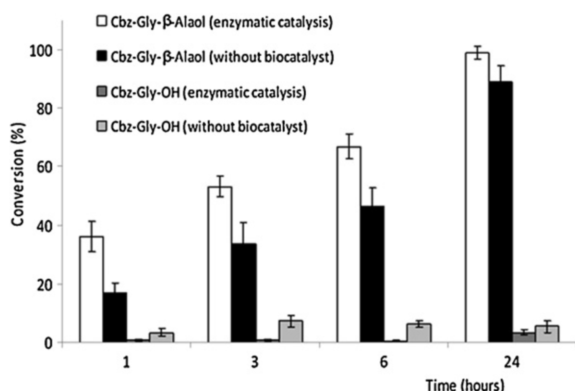


Fig. 1. Synthesis of Cbz-Gly-β-Alaol under kinetic control in presence and absence of biocatalyst.

Although synthesis under kinetic control is faster than the thermodynamically controlled one, the steps of derivatization of the acyl donor are also time-consuming. Furthermore, the use of extra chemicals for the activation reaction generates more waste, and thus the process becomes less clean. In this sense, the syntheses under thermodynamic approach proposed would be simpler and more according to the principles of eco-friendly technologies [51].

3.3. Biocatalytic synthesis of peptide alcohols using coded and non-coded amino acids as acyl donors using papain and araujiain as biocatalysts

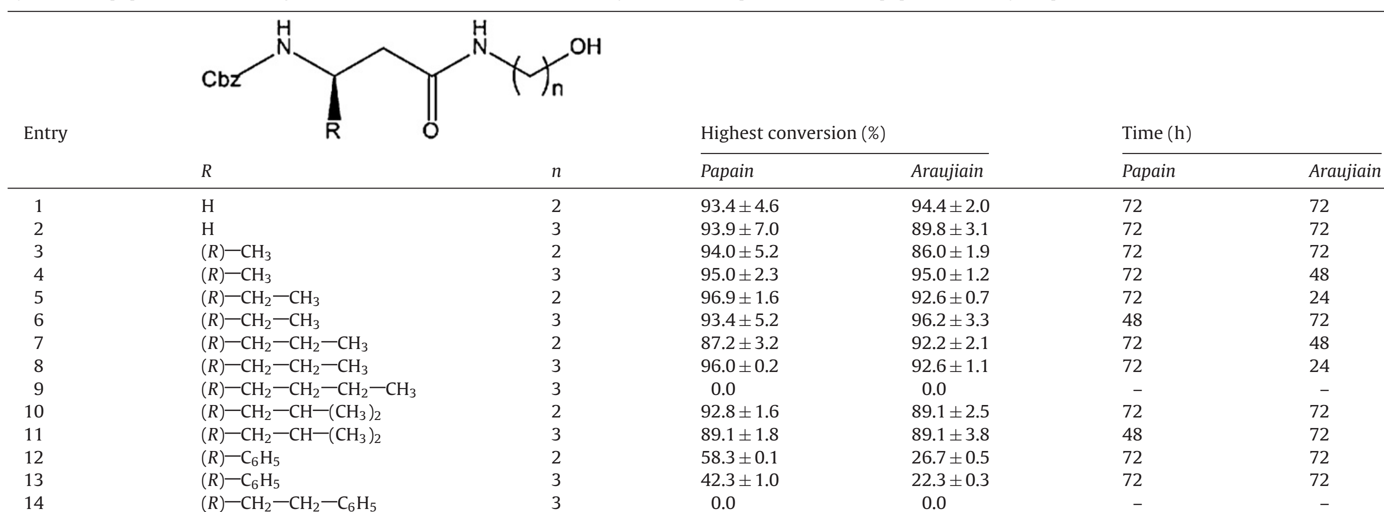
For the condensation reactions between Cbz-coded and non-coded amino acids and the two amino alcohols (ethanolamine and 3-amino-1-propanol), thermodynamically controlled synthesis was assayed. Papain and araujiain were tested as biocatalysts.

Reactions were performed in the same conditions described in the previous section. In general terms, both biocatalysts showed very similar performances in all cases, a fact that corroborates the biochemical similitude of the enzymes (Table 2). Fig. 2 shows the reaction progress for a typical synthesis using a coded amino acid derivative (Cbz-Leu-OH, Fig. 2A) and a non-coded one (Cbz-Nva-OH, Fig. 2B).

As can be observed in Table 2, both papain and araujiain could perfectly accept in their active sites the hydrophobic residues of increasing size of the acyl donors tested (in the range comprised by Gly and Leu residues). The limits seemed to be given by Nle and homoPhe, which residues may be too voluminous to fit in the S1 papain's subsite (Schechter and Berger nomenclature) [52]. This consists in an interesting finding, since the S1 subsite of papain is claimed to have a broad specificity for coded amino acids, there have been shown slight preferences for basic residues [53]; only Val is not accepted. In this sense, the S2 subsite is more selective for bulky non-polar residues [54–56]. On the other hand, conversions for the peptide alcohols containing Phg were considerably lower than those obtained for the other derivatives. This behaviour could be produced by the phenyl group of Phg, which would not be able to fit appropriately in the S1 subsite and thus, the (P1)C—NH—CO—C(P2) dihedral angle would not be the optimal for catalysis [57]. It is also noteworthy that papain showed higher conversions than araujiain for the syntheses concerning Phg derivative as acyl donor: this appears to be the most remarkable difference between the assayed biocatalysts. Other remarkable aspect is that araujiain reached the highest conversion rates in a lesser time than papain for the synthesis of the peptide alcohols containing Nva (24 h for Cbz-Nva-Glyol and 48 h for Cbz-Nva-β-Alaol).

The synthesis reactions of Cbz-Gly-Glyol, Cbz-Ala-Glyol and Cbz-Gly-β-Alaol were scaled up using papain onto polyamide as biocatalyst. The overall yields obtained after purification were 71.0, 93.3 and 94.3%, respectively. These good results indicate the strategy for synthesis and purification were simple and very appropriate, since the only impurities present in the final crude reaction mixture were, basically, the starting chemicals that did not react after reaching the maximum conversion and some hydrophobic material desorbed from the biocatalyst, as observed by TLC (data not shown). Analyses by NMR indicated that the enzyme formed selectively the amide bond between the amino acid derivative and

Table 2
Synthesis of peptide alcohols using coded and non-coded amino acids as acyl donors. Comparison between papain and araujiain performances.



Entry	*R*	*n*	Highest conversion (%)		Time (h)	
			Papain	*Araujiain*	*Papain*	*Araujiain*
1	H	2	93.4 ± 4.6	94.4 ± 2.0	72	72
2	H	3	93.9 ± 7.0	89.8 ± 3.1	72	72
3	(*R*)—CH_3	2	94.0 ± 5.2	86.0 ± 1.9	72	72
4	(*R*)—CH_3	3	95.0 ± 2.3	95.0 ± 1.2	72	48
5	(*R*)—CH_2—CH_3	2	96.9 ± 1.6	92.6 ± 0.7	72	24
6	(*R*)—CH_2—CH_3	3	93.4 ± 5.2	96.2 ± 3.3	48	72
7	(*R*)—CH_2—CH_2—CH_3	2	87.2 ± 3.2	92.2 ± 2.1	72	48
8	(*R*)—CH_2—CH_2—CH_3	3	96.0 ± 0.2	92.6 ± 1.1	72	24
9	(*R*)—CH_2—CH_2—CH_2—CH_3	3	0.0	0.0	–	–
10	(*R*)—CH_2—CH—$(CH_3)_2$	2	92.8 ± 1.6	89.1 ± 2.5	72	72
11	(*R*)—CH_2—CH—$(CH_3)_2$	3	89.1 ± 1.8	89.1 ± 3.8	48	72
12	(*R*)—C_6H_5	2	58.3 ± 0.1	26.7 ± 0.5	72	72
13	(*R*)—C_6H_5	3	42.3 ± 1.0	22.3 ± 0.3	72	72
14	(*R*)—CH_2—CH_2—C_6H_5	3	0.0	0.0	–	–

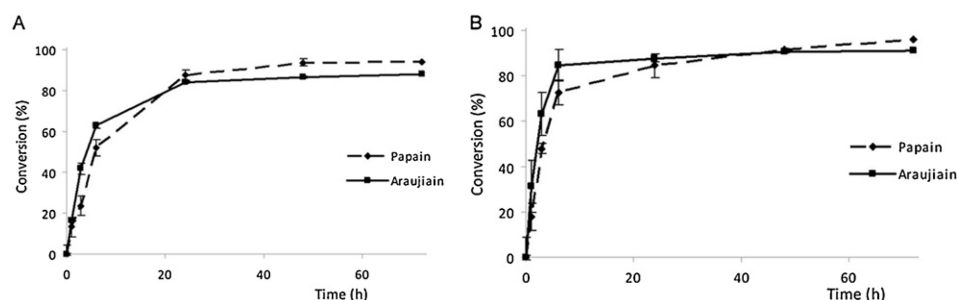


Fig. 2. Time-course reaction for the synthesis of (A) Cbz-Leu-Glyol and (B) Cbz-Nva-β-Alaol catalyzed by papain and araujiain.

the nucleophile, instead of acylating the –OH of the amino alcohol to obtain the ester, which is consistent with the conditions chosen for the reaction.

3.4. Synthesis of Cbz-Ala-β-alaninal diacetal derivative by condensation of Cbz-Ala-OH and 3,3-dietoxipropane-1-amine catalyzed by peptidases

Peptide aldehydes can be obtained by two main strategies namely the introduction of the aldehyde function on peptides, or using amino aldehydes conveniently protected as starting materials [8]. In the first approach, the strategy is based on the production of the peptide alcohol and the subsequent oxidation with different agents [8]. As it has been demonstrated in the previous section, peptide alcohols can be obtained via peptidase catalysis. In the second approach, the amino aldehyde can be introduced as a ketal derivative avoiding the cumbersome oxidation step. We tested the coupling of a donor with amino diacetals by protease catalysis for the preparation of dipeptide aldehyde derivatives. Reaction was carried out using a similar procedure to that for peptide alcohol synthesis, but using 3,3-dietoxypropane-1-amine as nucleophile (Fig. 3). According to the results (see Fig. 4), reaction was stopped as described in Section 2. When the assay was made in the usual way, i.e., by stopping the enzymatic reaction with AcH and analyzing the samples by HPLC–MS using mobile phases containing TFA (Fig. 4A), a minor peak with $t_R \cong 21$ min was identified as the expected product (Fig. 3), the Cbz-Ala-β-alaninal diacetal derivative. The peak with $t_R \cong 13$ min was identified as Cbz-Ala-β-alaninal and its hydrate. When the reaction was stopped by placing the vial in an ice-water bath, and a sample analyzed by HPLC–MS with TFA in the mobile phase (Fig. 4B), the peak with t_R of 21.9 min showed an increased area. Finally, when the same sample was analyzed without TFA in the mobile phase, the only peak observed corresponded to the Cbz-Ala-β-alaninal diacetal derivative (Fig. 4C).

As can be concluded after inspecting the chromatograms, the proteases produced the dipeptide diacetal derivative, which turned into the corresponding peptide aldehyde by acid catalysis. This acid catalysis was produced preferentially after adding AcH to stop the enzymatic reaction, and even took place in the chromatographic column, due to the presence of TFA in the mobile phase.

The results obtained would consist in a promising alternative to synthetize peptide aldehydes though enzymatic catalysis. The

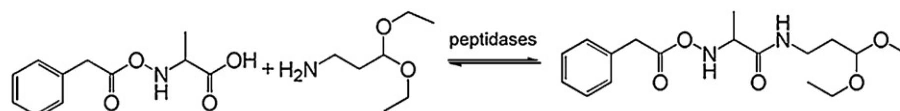


Fig. 3. Condensation reaction between Cbz-Ala-OH and 3,3-dietoxipropane-1-amine catalyzed by plant peptidases.

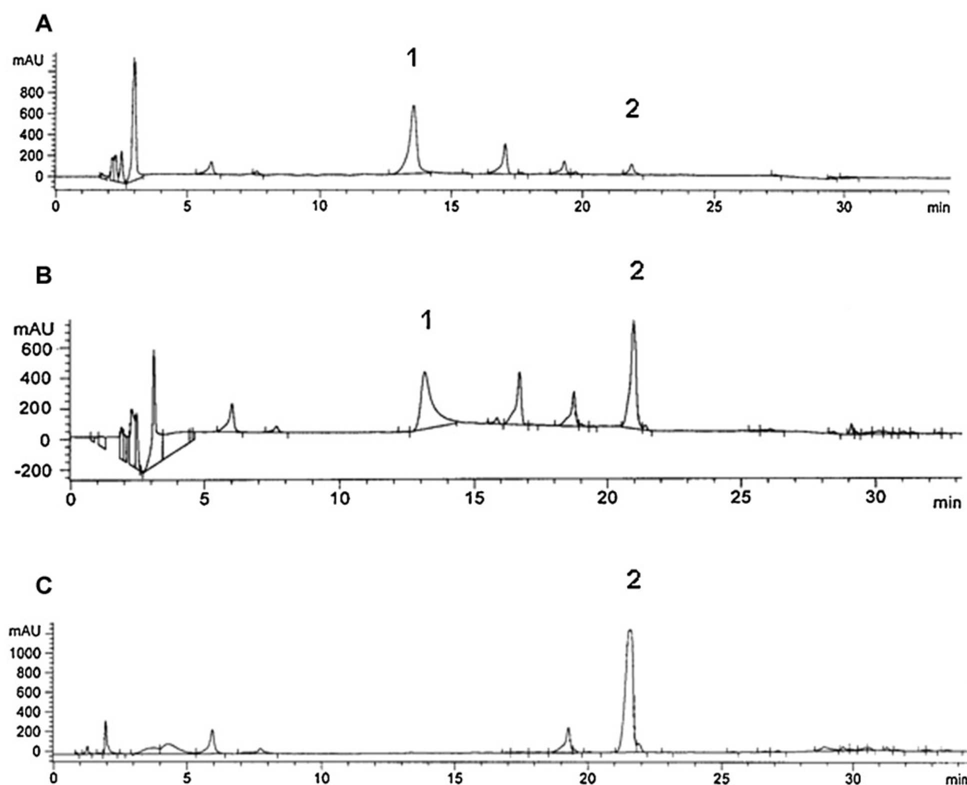


Fig. 4. Chromatograms obtained for the reaction shown in Fig. 3. (A) Reaction stopped with AcH and with TFA in the mobile phase. (B) Reaction stopped in an ice-water bath and with TFA in the mobile phase. (C) Reaction stopped in an ice-water bath and without TFA in the mobile phase. Peak 1, Cbz-Ala-β-alaninal and its hydrate; peak 2, Cbz-Ala-β-alaninal diacetal derivative.

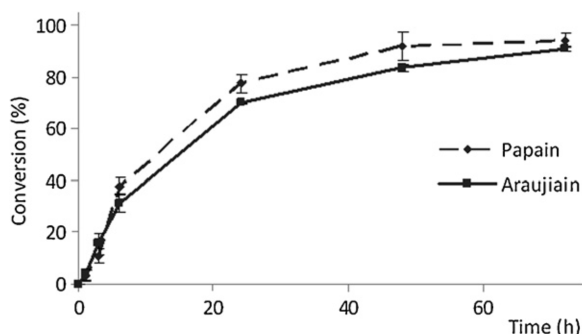


Fig. 5. Time-course reaction for the synthesis of the Cbz-Ala-β-alaninal diacetal derivative catalyzed by papain and araujiain.

product generated would not seem to work as a protease inhibitor as shown in Fig. 5, both biocatalysts reached a conversion of 90% in the condensation product after 72 h of reaction. This could be due to the protection of the aldehyde function as a diacetal, masking and turning it less reactive.

4. Conclusions

The synthesis of peptide alcohols containing coded and non-coded amino acids using papain and araujiain adsorbed onto polyamide as biocatalyst was achieved under thermodynamic controlled synthesis approach. The choice of the thermodynamic approach for the dipeptide derivatives syntheses was made basically after finding that the activated acyl donor tested (an -OCam derivative) rendered high conversions in product without the presence of the biocatalyst.

The conversions in product were comprised in the range of 85.0–96.0% for both enzymes, except for the case of Cbz-Phg-OH, in which yields were about 40–60% for papain and 25% for araujiain. Nle and homoPhe derivatives were not accepted as substrates by the biocatalysts in the condition tested.

Both enzymes showed a wide range of preferences in their S1′ subsites. This fact turn them into very versatile catalysts for acyl transfer to alcohols and amines as nucleophiles, which structures are different from those of peptides and amino acids. Papain has been proved to be a good catalyst for the acylation of diols and other alcohols [27,30], as well as keto- and lactam-amines [33,58]. In this report, this lack of specificity was exploited for the use of a simple amino diacetal, which demonstrated to be an excellent nucleophile for the synthesis, via enzymatic catalysis, of a dipeptide aldehyde precursor. In this sense, papain and araujiain showed a similar performance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.12.004.

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