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Comparative behaviour of proteinases from the latex of *Carica papaya* and *Funastrum clausum* as catalysts for the synthesis of Z-Ala-Phe-OMe

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Abstract

The proteolytic extract obtained from the latex of *Funastrum clausum* (Jacq.) Schlechter (*Asclepiadaceae*), a South American climbing plant, was assayed as a novel catalyst for peptide synthesis and compared with commercial papain under the same conditions. After immobilization on polyamide, the synthesis of the bitter peptide precursor Z-Ala-Phe-OMe was performed and different conditions were tried. Acetonitrile and ethyl acetate with low water content were tested as organic solvents. Equilibrium- and kinetically-controlled synthesis were tried by using either Z-Ala-OH or Z-Ala-OMe as acyl donors, respectively. The best conditions for the synthesis of the desired product varied according to the catalyst used. For papain, thermodynamic control in acetonitrile ($a_w \cong 0.12$) in the presence of triethylamine (TEA) or boric acid–borate buffer (40 mM), and equilibrium- and kinetic-controlled synthesis in ethyl acetate ($a_w \cong 0.75$) proved to be the best conditions. The thermodynamic control in either acetonitrile with $a_w \cong 0.12$ (40 mM TEA or Na_2CO_3) or ethyl acetate ($a_w \cong 0.75$) were the best conditions found for funastrain. In all cases, the formation of oligopeptides up to three Phe was observed. The proteolytic extract of *F. clausum* latex showed more selectivity than papain towards the conversion to Z-Ala-Phe-OMe leading to less proportion of oligopeptides.

Keywords: Protease catalysis; Funastrain; Papain; Peptide synthesis; Oligopeptides

1. Introduction

The ubiquity and consequence of proteases in nature, which represent approximately 2% of the genes in most organisms and are involved in virtually every physiological process, make them paradigms of enzyme catalysis [1]. They are also the most used enzymes in industry and biotechnology: peptidases account for almost the 50% of the market share [2]. The development of numerous biotechnological processes, which produce a wide range of important industrial commodities, has led to many efforts to harness protease catalytic power to many different applications, to produce special polymers, sweeteners and peptidic hormones [3,4]. These advances encourage the uncovering of new enzymes from multiple sources, which may prove industrially useful.

Papain is the most exploited plant protease in the brewing and baking industries [2]. It has been also successfully applied in synthesis of many compounds such as peptides [5], lipoamino acid-based surfactants [6], esters of amino acids [7] and carbohydrate derivatives [8]. Likewise, other papain-like peptidases could be applied as catalysts for similar reactions. The great biodiversity of South America [9,10] may be exploited as potentially useful source of biochemical catalysts for organic synthesis. As a matter of fact, from the latex of a local climbing milkweed *Morrenia brachystephana* Griseb. (*Asclepiadaceae*), a cysteine protease (morrenain b I) was purified [11], and proved to be a useful catalyst for peptide synthesis in an aqueous-organic biphasic system [12].

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Funastrum clausum (Jacq.) Schlechter (*Asclepiadaceae*), is a milkweed vine widely spread in tropical and subtropical areas of South America, especially in the North and central regions of Argentina [13]. Biochemical properties of funastrain and its principal endopeptidase, funastrain c II, revealed that they belong to the papain-like protease family [13,14], other milkweed proteinases recently studied [11,16–19].

The aim of this work is to compare the ability of both papain and funastrain, the crude proteolytic extracts obtained from the latex of *Carica papaya* and *F. clausum* (Jacq.) for the synthesis of the bitter dipeptide derivative Z-Ala-Phe-OMe.

2. Experimental

2.1. Materials

Funastrain, the crude extract from the latex of *F. clausum* (Jacq.) Schlechter (*Asclepiadaceae*), was obtained according to Morcelle et al. [14] and lyophilized for later immobilization. Specific activity of funastrain was determined by using N^{α}-benzoyl-L-arginine ethyl ester (BAEE, Sigma) as substrate [20], resulting in 2.5 BAEE units mg^{-1} of lyophilized powder (1 BAEE unit is defined as the enzyme activity that hydrolyzes 1.0 μmol of BAEE hydrochloride per min at pH 6.2 at 25 °C). Papain (EC 3.4.22.2) crude powder (1.3–3.5 BAEE units mg^{-1} of solid) was purchased from Sigma. Polyamide-6 (EP-700, particle size <800 μm, mean pore diameter 50–300 nm, specific surface area BET method 8.4 $m^2\ g^{-1}$) was an Azko (Obernburg, Germany) generous contribution. *N*-Benzyloxycarbonyl-L-alanine (Z-Ala-OH) and L-phenylalanine methyl ester hydrochloride (Phe-OMe.HCl) were supplied by Bachem (California, USA). *N*-Benzyloxycarbonyl-L-alanine methyl ester (Z-Ala-OMe) was synthetized by the tionyl chloride method [21]. L-Pyroglutamyl-L-phenylalanil-L-lecine *p*-nitroanilide (PFLNA) was synthetized in solid phase following the procedure described by Rivera et al. [22]. 1,4-Dithio-D,L-threitol (DTT) was provided by Fluka. The rest of the chemicals and solvents used in this work were of analytical grade.

2.2. Immobilization

Funastrain and papain were deposited onto polyamide [6] as follows. Each crude enzymatic powder (100 mg) and DTT (50 mg) was dissolved in boric acid–borate buffer 0.1 M pH 8.2 (1 mL) and mixed with the support (1 g). After mixing vigorously, the resulting mixture was evaporated under vacuum (approximately 40 μbar) for 24 h.

2.3. Determination of remaining enzymatic activity of immobilized preparations

The activity of papain- and funastrain-polyamide catalysts was determined after drying using PFLNA as substrate, according to Filippova et al. [23]. Measurements were made on 10 mg of each immobilized preparation in 0.1 M phosphate buffer (pH 6.5) containing 0.3 M KCl, and 10^{-4} M EDTA. PFLNA in dimethyl sulfoxide (DMSO) was added to the mixture, giving a substrate final concentration of 0.15 mM. Incubation was performed at 37 °C for 5 min in an orbital shaker (150 rpm). The reactions were stopped by the addition of 0.5 mL of 30% AcH. Each tube was centrifuged 20 min at 4000 rpm to separate the immobilized preparations. The absorbance of the *p*-nitroaniline released in the supernatant was measured spectrophotometrically at 410 nm. Similar determinations were made for the free enzymes, but adding DTT to the reaction buffer in the same proportion as in the immobilized preparations to assure the same reaction conditions. Enzymatic units (U_{PFLNA}, defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline per min in the assay conditions) were obtained by performing a standard curve of *p*-nitroaniline.

2.4. Enzymatic synthesis of Z-Ala-Phe-OMe

All the assays were carried out in vials containing immobilized enzyme (200 mg), and the solvent (2 mL) under argon atmosphere, and placed on a reciprocal shaker (120 rpm) at 25 °C. As acyl donors Z-Ala-OH (30 mM) and Z-Ala-OMe (30 mM) were used in equilibrium- and kinetically-controlled synthesis, respectively, whereas Phe-OMe (50 mM) was proved as nucleophile in every case. Samples (50 μL) were withdrawn at 1, 3, 6, 24, 48 and 72 h, from the reaction mixture supernatant and acetic acid (10 μL) were added to stop the reaction for further HPLC analysis as described below.

2.5. Enzymatic synthesis of Z-Ala-Phe-OMe in acetonitrile

The reactions were performed by the general experimental procedure described above in acetonitrile containing 0.5% (v/v) water ($a_w \cong 0.12$) and triethylamine (TEA), boric acid–borate buffer pH 9.0 or $NaHCO_3$ (40 mM in all cases) depending on the experiment.

2.6. Enzymatic synthesis of Z-Ala-Phe-OMe in ethyl acetate

The reactions were performed by the general experimental procedure described above in ethyl acetate at approximately, a_w 0.75 as solvent system for both synthetic approaches. The following procedure was performed in order to have the nucleophile in free base form. A stock aqueous solution of PheOMe.HCl (50 mM) was neutralized with Na_2CO_3 (2 equiv/mol) and extracted twice in ethyl acetate.

2.7. HPLC analysis and product characterization

Reactions were monitored by HPLC (Merck-Hitachi Lichrograph System, Darmstadt, Germany) using a Lichrospher 100 RP-18, 5 μm, 250 mm × 4 mm column (Merck). The chromatographic conditions were the following: solvent A, acetic acid 0.5% (v/v) in water; solvent B, acetic acid 0.5% (v/v) in H_2O:CH_3CN 20:80; elution gradient 40–100% B in 40 min; flow rate, 1 mL/min; detection 254 nm. Quantitative analysis was performed from peak areas by means of external standard method for the substrate and dipeptide. From the calibration curves of both substrate and dipeptide, the calibration curves of the oligomerization products were extrapolated considering

that the peak areas were approximately proportional to the number of Phe in the molecule. Specific productivity (defined as q_p = nmoles of Z-Ala-Phe-OMe/enzyme unit × h) was calculated in each case by means of the external standard method. Product characterization was accomplished by HPLC mass spectrometry.

3. Results and discussion

Due to the biochemical similarities between funastrain and papain [14,15], both proteases were tested and compared as catalysts for the same peptide formation under the same conditions. The chosen product was the bitter dipeptide precursor Z-Ala-Phe-OMe for the following reasons: (a) the potential interest of the product for the food industry [24]; (b) funastrain highest preference for the Ala derivative in assays using *p*-nitrophenyl esters of *N*-protected amino acids as substrates [14]. Z-Ala-OH and Z-Ala-OMe (30 mM) were chosen as acyl donors for equilibrium- and kinetically-controlled synthesis, respectively, and Phe-OMe.HCl (50 mM) was the nucleophile. A polar organic media consisting of acetonitrile with 0.5% (v/v) water ($a_w \cong 0.12$) and a non polar one consisting of ethyl acetate with $a_w \cong 0.75$ were selected as the two reaction media, because they have proved to be excellent reaction systems for both, substrate solubility, and enzyme activity and stability. Furthermore, both solvents gave the best results in peptide bond formation catalyzed by either papain or α-chymotrypsin [25]. Deposited enzymes onto solid supports were the catalysts configuration chosen for these experiments. Polyamide was the selected support material mostly because it has been widely demonstrated that is a very appropriate material to deposit peptidases for amide and ester bond formation [6]. Residual enzymatic activity of the biocatalysts in aqueous media was measured after immobilization using PFLNA as substrate. The remaining amidolytic activity was 25% for papain and 23% for funastrain with regard to the initial activity of free enzymes, showing similar yield of immobilization as well as similar denaturation degree during the whole process.

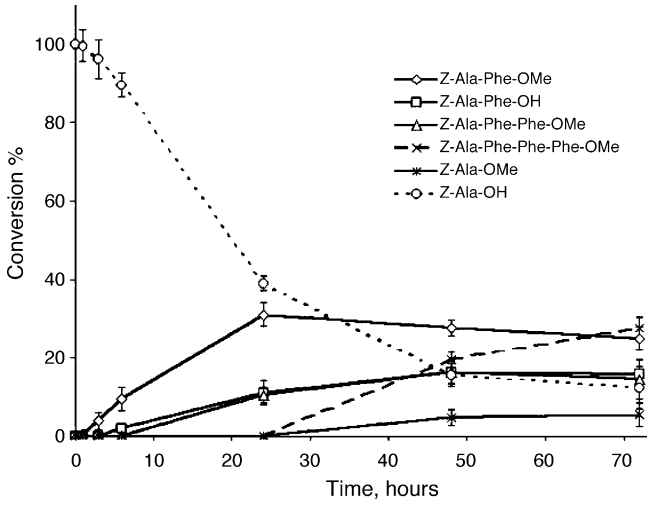

Fig. 2. Time-course reaction curves for equilibrium-controlled synthesis using papain as catalyst in acetonitrile $a_w \cong 0.12$.

Since the Phe-OMe.HCl is not reactive, the effective nucleophile concentration (Phe-OMe) was increased by adding different alkalis. To achieve this purpose, a reaction was developed using TEA (40 mM) and papain as catalyst in acetonitrile with 0.5% (v/v) water, under equilibrium-controlled synthesis. A visual inspection of the reaction medium revealed an increasing development of a turbidity with the time which end up to a final white precipitate. The HPLC peak profile (Fig. 1) and HPLC-mass spectrometry analysis of the samples at different reaction time, indicated that, apart from the dipeptide (Z-Ala-Phe-OMe), the formation of polymerization products such as the tripeptide, Z-Ala-$(Phe)_2$-OMe, and the tetrapeptide, Z-Ala-$(Phe)_3$-OMe, as well as the esterification of the Z-Ala-OH, occurred probable due to the MeOH released during other acylation reactions. The white precipitated collected at the end of the reaction was

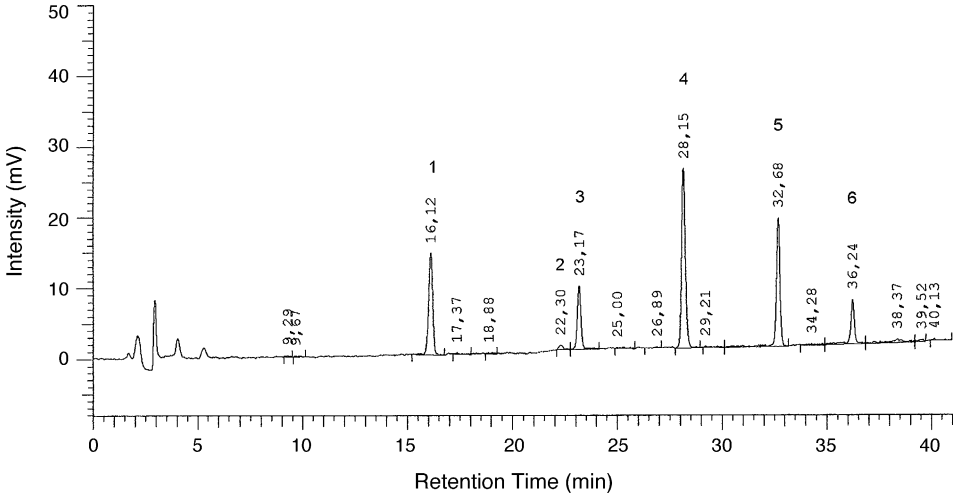

Fig. 1. Chromatographic peak profile after 24 h reaction (papain in acetonitrile $a_w \cong 0.12$, thermodynamic control synthesis). 1, Z-Ala-OH; 2, Z-Ala-OMe; 3, Z-Ala-Phe-OH; 4, Z-Ala-Phe-OMe; 5, Z-Ala-$(Phe)_2$-OMe; 6, Z-Ala-$(Phe)_3$-OMe. *Mass Spectrometry results*. Z-Ala-Phe-OMe ESMS [$M+1$] 385.3; [Z-Ala-Phe-OMe requires 384.4]; Z-Ala-Phe-OH ESMS [$M+1$] 371.3 [Z-Ala-Phe-OH requires 370.4]; Z-Ala-$(Phe)_2$-OMe ESMS [$M+1$] 532.4 [Z-Ala-$(Phe)_2$-OMe requires 531.6]; Z-Ala-$(Phe)_3$-OMe ESMS [$M+1$] 679.6 [Z-Ala-$(Phe)_3$-OMe requires 678.6]; Z-Ala-OMe ESMS [$M+23$] 260.2 [Z-Ala-OMe requires 237.2].

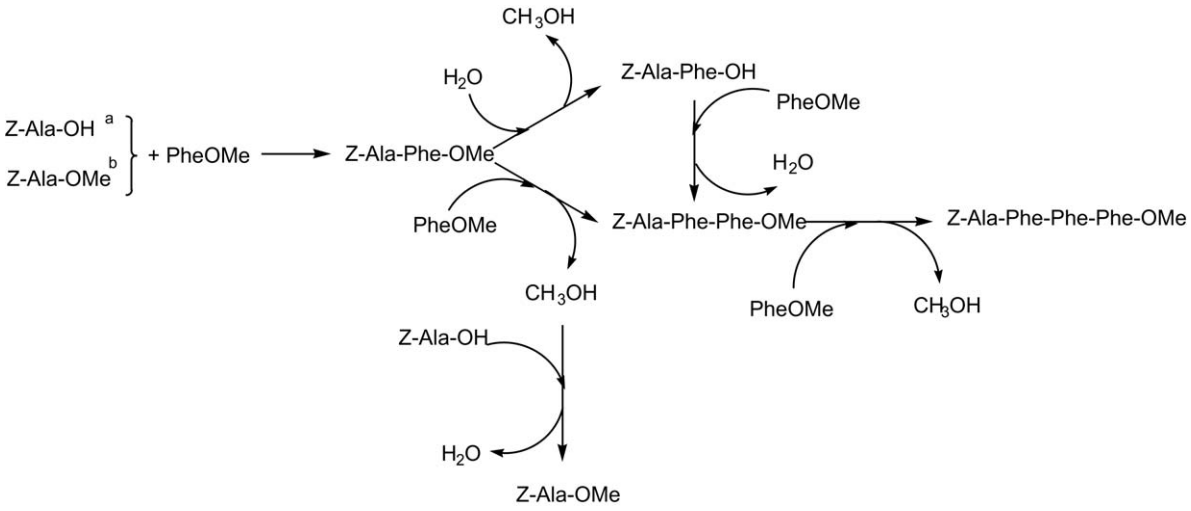

Fig. 3. Proposed steps for the formation of the different products catalyzed by the proteases. a, Thermodynamic control and b, kinetic control.

identified as the tetrapeptide. The typical time-course reaction plot, shown in Fig. 2, reflects the formation of the different enzymatic condensation products. In an early study, Sluyterman and Wijdenes analyzed the activity of papain with Leu-OMe acting as acyl donor and nucleophile. They observed that the catalytic action of papain on this substrate produced insoluble polyleucine peptides, typically from eight to nine residues, which appeared as a white opalescence in the reaction medium. They also reported similar results when Phe-OMe was used as substrate after standing overnight [26]. According to this, Phe-OMe could act not only as nucleophile but as acyl donor itself. Bearing this in mind, one of the possible products of the reaction could have been a polymer based on Phe. Nevertheless, this was not observed for papain or for funastrain under the conditions used in this work. This is in agreement with the observation that the specificity at the S_1 subsite of papain is stronger for Ala than for Phe [27].

The formation of these oligopeptides may be due to the esterolytic activity of the proteases used for these syntheses. This esterolytic activity is reinforced by the reaction pH, at alkaline pH papain shows esterolytic activity a thousand times higher than amidolytic [28]. This is a convenient property for peptide synthesis, because product hydrolysis should be negligible. However, in this case, the desired product Z-Ala-Phe-OMe, would act as an activated acyl donor, which would then react with Phe-OMe, giving the tri-, and tetrapeptide derivatives or with water, leading to the hydrolysis product, Z-Ala-Phe-OH (Fig. 3). When papain was assayed as catalyst for aspartame synthesis, a similar result was reported [29]. The correlation between the formation of strongly hydrophobic polymers and the development of turbidity, reminds of the plastein reaction. The plastein reaction has been defined, in general terms, as the conversion, in presence of a peptidase, of freely soluble concentrated solutions of peptides into reaction mixtures containing a large amount of material that is insoluble in either aqueous buffer, organic solvents and after alkali or acidic treatment [30]. Transpeptidation reactions have been reported as the responsible events for this phenomenon [30,31]. The formation of this kind of compounds may be of interest for food industry as gels or proteinaceus surfactants [32].

The results obtained thus far have shown that different peptide products are obtained starting from two simple amino acid derivatives. To obtain reaction conditions for producing each of them selectively, the reaction conversion to the products was investigated for the following variables: (i) alkali used for the neutralization of Phe-OMe, (ii) the synthetic approach (i.e. thermodynamically or kinetically-controlled synthesis) and (iii) organic media. All these variables were studied for both enzymes.

The effect of three different basic compounds, TEA, boric acid–borate buffer pH 9.0 and Na_2CO_3 was investigated (Tables 1–3). TEA was the alkali that gave the highest con-

Table 1
Synthesis of Z-Ala-Phe-OMe under thermodynamic control using TEA 40 mM in acetonitrile $a_w \cong 0.12$

Time (h)	Conversion (%)										Unreacted substrate (%)	
	Z-Ala-Phe-OMe		Z-Ala-Phe-OH		Z-Ala-(Phe)$_2$-OMe		Z-Ala-(Phe)$_3$-OMe		Z-Ala-OMe		Z-Ala-OH	
	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain
1	0	5 ± 1	0	0	0	0	0	0	0	0	100 ± 4	95 ± 1
3	4 ± 2	8 ± 2	0	0	0	0	0	1 ± 1	0	0	95 ± 5	91 ± 4
6	9 ± 3	15 ± 2	2 ± 1	1 ± 1	0	1 ± 1	0	2 ± 2	0	0	89 ± 3	81 ± 3
24	31 ± 3	23 ± 4	11 ± 3	5 ± 3	15 ± 2	3 ± 2	4 ± 2	4 ± 1	0	0	39 ± 2	65 ± 2
48	28 ± 2	24 ± 2	16 ± 3	9 ± 3	16 ± 3	1 ± 1	19 ± 3	5 ± 2	5 ± 2	3 ± 2	16 ± 3	58 ± 5
72	25 ± 3	23 ± 3	16 ± 2	12 ± 2	14 ± 5	2 ± 1	27 ± 5	2 ± 1	5 ± 3	5 ± 3	13 ± 5	56 ± 2

Table 2
Synthesis of Z-Ala-Phe-OMe under thermodynamic control using boric acid–borate buffer 40 mM pH 9.0 in acetonitrile $a_w \cong 0.12$

Time (h)	Conversion (%)										Unreacted substrate (%)	
	Z-Ala-Phe-OMe		Z-Ala-Phe-OH		Z-Ala-(Phe)$_2$-OMe		Z-Ala-(Phe)$_3$-OMe		Z-Ala-OMe		Z-Ala-OH	
	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain
1	0	0	0	0	0	0	0	0	0	0	100 ± 3	100 ± 2
3	0	0	0	0	0	0	0	0	0	0	100 ± 2	100 ± 3
6	1 ± 1	1 ± 1	16 ± 3	0	0	0	0	0	0	0	83 ± 4	99 ± 4
24	15 ± 3	10 ± 3	10 ± 3	0	2 ± 1	1 ± 1	0	1 ± 1	0	0	52 ± 5	88 ± 5
48	28 ± 2	16 ± 5	10 ± 3	0	10 ± 2	2 ± 1	2 ± 1	4 ± 1	0	0	50 ± 3	78 ± 3
72	32 ± 5	21 ± 6	16 ± 2	1 ± 1	19 ± 4	2 ± 1	5 ± 3	6 ± 1	0	0	28 ± 4	70 ± 4

Table 3
Synthesis of Z-Ala-Phe-OMe under thermodynamic control using Na_2CO_3 40 mM as alkali in acetonitrile $a_w \cong 0.12$

Time (h)	Conversion (%)										Unreacted substrate (%)	
	Z-Ala-Phe-OMe		Z-Ala-Phe-OH		Z-Ala-(Phe)$_2$-OMe		Z-Ala-(Phe)$_3$-OMe		Z-Ala-OMe		Z-Ala-OH	
	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain
1	3 ± 1	6 ± 2	0	0	1 ± 1	0	2 ± 1	0	0	0	94 ± 4	94 ± 5
3	8 ± 2	10 ± 3	1 ± 1	0	4 ± 2	0	0	0	0	0	87 ± 3	90 ± 2
6	13 ± 1	16 ± 2	4 ± 2	1 ± 1	9 ± 3	3 ± 2	3 ± 1	0	0	0	55 ± 5	80 ± 3
24	16 ± 4	24 ± 3	3 ± 2	5 ± 2	24 ± 3	3 ± 1	14 ± 2	9 ± 2	9 ± 2	0	34 ± 3	59 ± 4
48	15 ± 3	25 ± 4	11 ± 3	9 ± 2	13 ± 2	4 ± 2	18 ± 2	8 ± 3	13 ± 3	0	30 ± 5	54 ± 4
72	7 ± 2	19 ± 3	7 ± 3	12 ± 3	3 ± 1	5 ± 2	56 ± 4	20 ± 2	11 ± 2	0	16 ± 4	44 ± 3

version to the dipeptide (22–23%) at the shortest reaction time (24 h) for both funastrain and papain (Table 1). Using papain, boric acid–borate buffer gave 32% conversion to Z-Ala-Phe-OMe after 72 h (Table 2) but with low selectivity (i.e. 40% of by-products) (Table 2). Funastrain rendered the best Z-Ala-Phe-OMe conversions (25%) using Na_2CO_3, whereas under these conditions papain gave the tetrapeptide derivative as the major product (56%) in 72 h (Table 3). It is noteworthy that funastrain gave always the lowest amounts of oligopeptide derivatives, showing better selectivity than papain towards the synthesis of Z-Ala-Phe-OMe.

Modification of the reaction conditions included the use of the activated acyl donor and/or immiscible media. Due to the slow rate of the thermodynamically-controlled synthesis (i.e. reaction of Z-Ala-OH and Phe-OMe), and the fast incorporation of subsequent molecules of Phe-OMe (Fig. 2), the kinetically-controlled synthesis could prevent or modify the polymerization reaction profiles. The progress of kinetic-controlled synthesis was followed up to 72 h (Table 4). With papain the major product after 24 h of incubation was the dipeptide (25%) but with low selectivity (28% of by products), while at 72 h 43% of the tetrapeptide was obtained. Thus, at longer times, the formation of tri- and tetrapeptide was as much as in the thermodynamically-controlled condition. As the donor ester was not completely consumed, it seems likely that Z-Ala-Phe-OMe was a better substrate for papain than Z-Ala-OMe, and when Z-Ala-Phe-OMe was produced to a certain amount, the driving force to the reaction was the formation of Z-Ala-(Phe)$_3$-OMe (Fig. 3).

A different behaviour was observed during the synthesis of the dipeptide with funastrain-polyamide catalyst under kinetic control conditions in acetonitrile. The reaction stopped after 1 h at *c.a.* 10% Z-Ala-Phe-OMe conversion, suggesting a fast deactivation of the enzyme under these conditions (Table 4). Interestingly, this deactivation did not occur under thermodynamically-controlled conditions. This might be due, among other things, to changes in the ionization state of the enzyme by the acyl donor

Table 4
Synthesis of Z-Ala-Phe-OMe under kinetic control in acetonitrile $a_w \cong 0.12$

Time (h)	Conversion (%)										Unreacted substrate (%)	
	Z-Ala-Phe-OMe		Z-Ala-Phe-OH		Z-Ala-(Phe)$_2$-OMe		Z-Ala-(Phe)$_3$-OMe		Z-Ala-OH		Z-Ala-OMe	
	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain
1	9 ± 2	10 ± 3	2 ± 2	1 ± 1	1 ± 1	1 ± 1	0	2 ± 1	4 ± 2	0	84 ± 3	86 ± 2
3	9 ± 1	10 ± 2	1 ± 1	1 ± 1	1 ± 1	1 ± 1	0	2 ± 1	2 ± 1	3 ± 1	87 ± 2	83 ± 3
6	12 ± 3	10 ± 2	2 ± 1	1 ± 1	2 ± 1	2 ± 1	0	2 ± 1	3 ± 2	2 ± 1	81 ± 4	83 ± 2
24	25 ± 3	14 ± 3	9 ± 2	2 ± 1	14 ± 3	14 ± 2	5 ± 2	3 ± 2	7 ± 2	3 ± 1	40 ± 5	64 ± 4
48	20 ± 3	14 ± 3	9 ± 3	3 ± 2	13 ± 3	13 ± 2	35 ± 5	3 ± 1	7 ± 1	6 ± 2	16 ± 3	61 ± 3
72	19 ± 2	14 ± 2	9 ± 2	0	11 ± 4	11 ± 3	43 ± 5	7 ± 3	6 ± 3	7 ± 1	12 ± 2	61 ± 2

Table 5
Synthesis of Z-Ala-Phe-OMe under kinetic control in ethyl acetate $a_w \cong 0.75$

Time (h)	Conversion (%)										Unreacted substrate (%)	
	Z-Ala-Phe-OMe		Z-Ala-Phe-OH		Z-Ala-(Phe)$_2$-OMe		Z-Ala-(Phe)$_3$-OMe		Z-Ala-OH		Z-Ala-OMe	
	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain
1	1 ± 1	3 ± 1	0	0	0	0	0	0	2 ± 1	0	96 ± 3	97 ± 4
3	1 ± 1	7 ± 3	0	0	0	0	0	0	2 ± 2	0	96 ± 2	93 ± 2
6	3 ± 2	13 ± 2	0	0	0	1 ± 1	0	1 ± 1	1 ± 1	4 ± 2	96 ± 4	81 ± 3
24	11 ± 3	21 ± 4	0	3 ± 1	2 ± 1	3 ± 2	0	3 ± 1	2 ± 1	10 ± 3	87 ± 3	60 ± 4
48	17 ± 2	18 ± 3	2 ± 1	7 ± 3	13 ± 4	2 ± 1	10 ± 2	2 ± 1	4 ± 2	21 ± 2	54 ± 4	50 ± 5
72	20 ± 5	15 ± 4	5 ± 2	9 ± 3	25 ± 5	3 ± 1	10 ± 3	2 ± 2	8 ± 3	29 ± 3	33 ± 3	42 ± 4

Table 6
Synthesis of Z-Ala-Phe-OMe under thermodynamic control in ethyl acetate $a_w \cong 0.75$

Time (h)	Conversion (%)										Unreacted substrate (%)	
	Z-Ala-Phe-OMe		Z-Ala-Phe-OH		Z-Ala-(Phe)$_2$-OMe		Z-Ala-(Phe)$_3$-OMe		Z-Ala-OMe		Z-Ala-OH	
	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain	Papain	Funastrain
1	2 ± 1	6 ± 1	0	0	0	0	0	0	0	0	98 ± 2	94 ± 3
3	1 ± 1	19 ± 2	0	0	0	0	0	0	0	0	99 ± 2	81 ± 2
6	22 ± 2	23 ± 4	0	1 ± 1	0	1 ± 1	0	1 ± 1	0	0	78 ± 3	74 ± 3
24	24 ± 2	24 ± 2	4 ± 2	9 ± 2	8 ± 2	1 ± 1	4 ± 2	2 ± 2	0	10 ± 2	60 ± 5	53 ± 2
48	21 ± 3	15 ± 3	7 ± 2	14 ± 2	23 ± 4	1 ± 1	17 ± 2	0	4 ± 2	17 ± 3	25 ± 2	52 ± 2
72	23 ± 2	14 ± 2	13 ± 2	16 ± 2	15 ± 3	1 ± 1	14 ± 3	1 ± 1	14 ± 4	18 ± 2	21 ± 3	51 ± 2

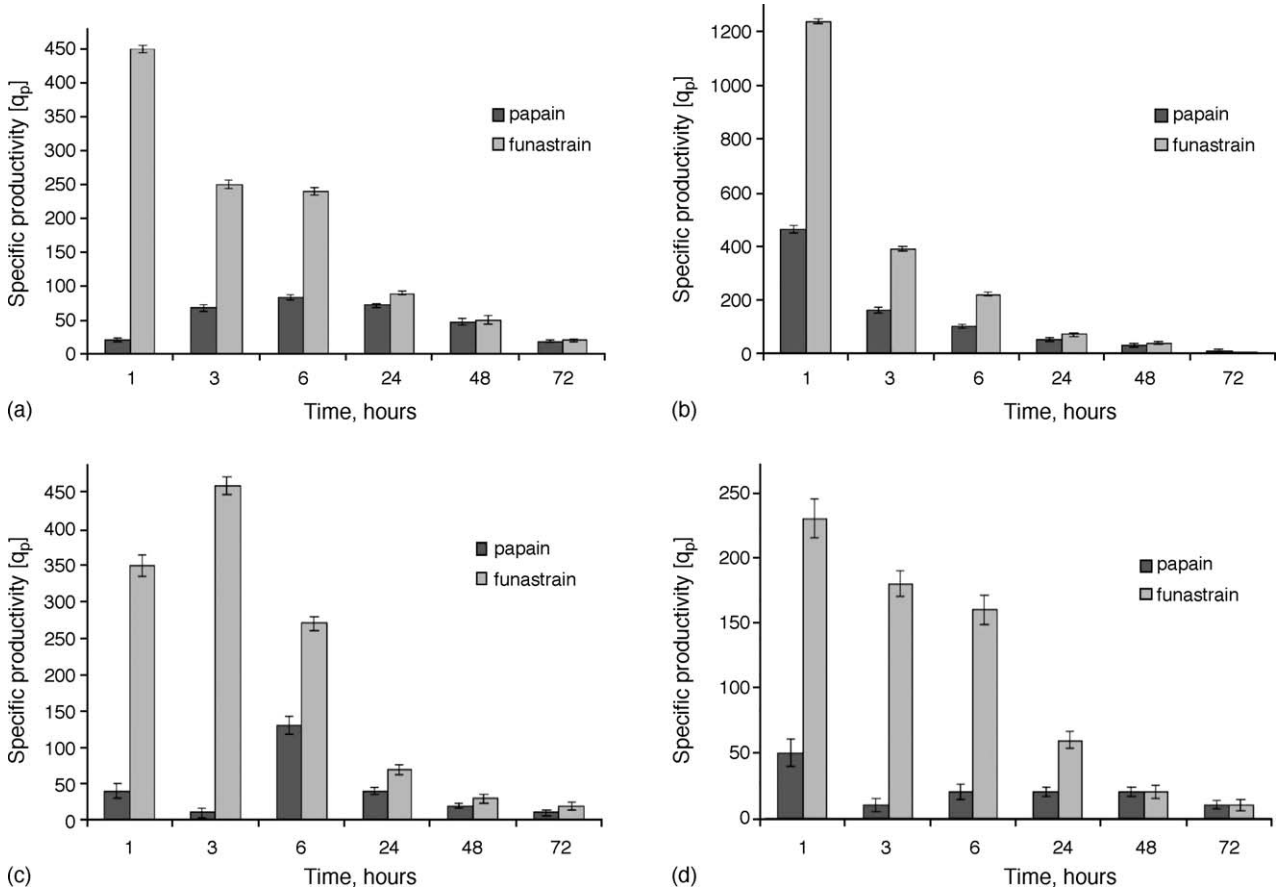

Fig. 4. Comparison of funastrain and papain specific productivities [q_p = nmoles of Z-Ala-Phe-OMe/enzyme unit × h] during the reaction course. (A) Equilibrium-controlled synthesis in acetonitrile of $a_w \cong 0.12$; (B) kinetic-controlled synthesis in acetonitrile of $a_w \cong 0.12$; (C) equilibrium-controlled synthesis in ethyl acetate of $a_w \cong 0.75$; (D) kinetic-controlled synthesis in ethyl acetate of $a_w \cong 0.75$.

substrate (i.e. acid or methyl ester). It should be noticed that when ethyl acetate was used as solvent (see Table 5) changes in the solvation of the reaction substrates, products and salts in both the enzyme microenvironment and bulk solvent might prevent the enzyme deactivation.

The effect of the solvent was also investigated. It was thought that increasing the solvent hydrophobicity the solvation effect on substrate and product might change the reaction profile. For this purpose, ethyl acetate at $a_w \cong 0.75$ was proved as an immiscible system for both equilibrium- and kinetically-controlled synthesis. Under thermodynamic control, both funastrain and papain gave 22–23% Z-Ala-Phe-OMe conversion (Table 6) at 6 h with high selectivity; no by-products were formed with papain and only 3% with funastrain. It is noteworthy that for longer incubation times funastrain gave conversions to Z-Ala-Phe-OH and Z-Ala-OMe higher than that to tri- and tetrapeptide, while the contrary is true for papain. For the kinetic control, the rate of Z-Ala-Phe-OMe conversion was much slower than expected, even slower than those for equilibrium-controlled synthesis for both catalysts. The reason for this behaviour could be attributed to differences in Z-Ala-OMe and Z-Ala-OH solvation in the aqueous microenvironment of the enzyme and the organic solvent. However, the product distribution did not changed substantially compared with the thermodynamic approach. Interestingly, funastrain furnished 21% conversion to Z-Ala-Phe-OMe with a remarkable selectivity performing, in this case, better than papain.

Overall, funastrain proved to be more specific than papain for the synthesis of Z-Ala-Phe-OMe, showing in general a higher ratio of conversion for this product. This is clearly evidenced by the fact that the specific productivity values for Z-Ala-Phe-OMe formation of funastrain were higher than the those of papain in short reaction times (Fig. 4). Among all the by-products formed by funastrain catalyzed reaction, those coming from hydrolysis were quantitatively the most important (mainly, Z-Ala-Phe-OH and, in the kinetic-controlled synthesis, Z-Ala-OH). It was stated that the formation of oligopeptides containing Phe residues by papain catalysis was probably due to its marked preference for large hydrophobic amino acid residues in the P_1' position [33]. Interestingly, this could be a difference with funastrain behaviour, which produced less polyamino acid peptides than papain. Although being a papain-like peptidase, funastrain could not share these characteristics, which makes it a preferable catalyst for the formation of the Z-Ala-Phe derivative.

4. Conclusions

Papain is, undoubtely, the most exploited plant endopeptidase in the food industry. In this paper an enzymatic extract from the latex of *F. clausum* (Jacq.) Schlechter (*Asclepiadaceae*), funastrain, is reported as an alternative catalyst belonging to the papain-like family of peptidases, in the less explored field of peptide synthesis. For the production of the bitter dipeptide precursor Z-Ala-Phe-OMe, both enzymes showed, in general terms, a similar behaviour due to their similar nature. Nevertheless, for the synthesis of this compound in particular, funastrain proved to be more specific than papain. This is substained by the higher yields in the desired product up to 6 h of reaction course obtained for funastrain in the four conditions tested (equilibrium-controlled synthesis and kinetically-controlled synthesis, either in miscible and immiscible media). The formation of different by-products (especially, polimerization products) was also much lower for the funastrain-catalyzed reaction than for papain. The most promising conditions for funastrain were those of equilibrium-controlled synthesis in acetonitrile with 0.5% water ($a_w \cong 0.12$) with either 40 mM TEA or Na_2CO_3 as alkalis, and equilibrium-controlled synthesis in ethyl acetate of $a_w \cong 0.75$. These conditions could be explored in further studies, by using a Z-Ala derivative more activated as acyl donor and an amide instead of ester derivative as nucleophile by comparing either funastrain and papain as biocatalysts.

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