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Peptide synthesis in aqueous–organic biphasic systems catalyzed by a protease isolated from *Morrenia brachystephana* (*Asclepiadaceae*)

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Abstract

A new cysteine protease, morrenain b II, isolated from the latex of a South American climbing plant, *Morrenia brachystephana* Griseb. (*Asclepiadaceae*), was found to be stable in aqueous–organic biphasic systems. In this work, we have investigated the ability of morrenain b II to perform peptide synthesis using Phe.OMe and Asp as substrates; Cys as activator; 0.1 M Tris–HCl buffer pH 8.5 and chloroform as reaction media. The reaction products were separated by RP-HPLC and identified by TLC, H-NMR and EI-MS. Morrenain b II showed high specificity towards bonds between Cys and Phe.OMe amino acids. A significant amount of the cystine–Phe.OMe peptide was produced within 1 h. These preliminary results were compared with papain under the same conditions.

Keywords: Cysteine proteases; Non conventional media; Peptide synthesis; *Morrenia brachystephana*

1. Introduction

Most enzyme catalytic processes are carried out in aqueous solutions. However, many reaction products cannot be obtained in aqueous media for various reasons such as insolubility of substrates, unfavorable thermodynamic equilibria, difficult product recovery and enzyme inhibition by reagents and/or products. In many cases, difficulties can be overcome by performing the reactions in water–organic solvent binary mixture [1].

In the growing field of preparative synthesis of peptides, enzymatic peptide synthesis, using proteases as catalyst, has become an integrated part of the process, valuable as a technique per se and as a tool for fragment condensation [2].

Enzymatic synthesis can usually proceed very specifically, without racemization and protection of reactants side chains [3]. Thus, a central issue in the development of this technology is the search for proteases with very well defined specificities. The two main advantages of enzymatic peptide synthesis are the high specificity of the reaction and the absence of any side chain protection requisite. In the development of the synthesis process, attention has been focused on dipeptides, due to their increasing importance in areas such as food flavoring, new pharmaceutical

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developments, agrochemistry and animal nutrition [4].

Among the various enzymes studied, the cysteine proteases papain and ficin have proved to be versatile biocatalysts for the synthesis of a great variety of peptides. The successful application of both enzymes raises the possibility that other cysteine proteases of plant origin can likewise be used as biocatalysts in peptide synthesis procedures [5].

The isolation, purification and characterization of a cysteine protease, morrenain b II, from the latex of *Morrenia brachystephana* Griseb. (a South American climbing plant) have been described previously [6].

In this paper, we report the application of morrenain b II to perform the peptide synthesis using an aqueous–organic biphasic systems.

2. Materials and methods

2.1. Materials

Morrenain b II was extracted and purified from latex obtained from petioles and stems of *M. brachystephana* (*Asclepiadaceae*) by a simple procedure involving acetone fractionation and cation exchange chromatography on a Sepharose CL-6B Fast Flow, according to Vairo Cavalli et al. [6]. Specific activity of morrenain b II was 8.8 Ucas/mg of protein. Commercial papain (E.C.3.4.22.2., 30000 USP/Umg) was supplied by E. Merck (Darmstadt, FRG).

2.2. Caseinolytic activity measurement

Proteolytic assays were performed using casein (Hammarsten type, Research Organics, Cleveland, OH, USA) as substrate. The reaction mixture was prepared by mixing 0.1 ml of the enzyme sample with 1.1 ml of 1% casein containing 12 mM Cys, in a 0.1 M Tris–HCl buffer pH 8.0. The reaction was carried out at 40 °C and it was stopped 6 min later by the addition of 1.8 ml of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at 3000 × *g* for 30 min and the absorbance of the supernatant was measured at 280 nm. An arbitrary enzyme unit (caseinolytic unit, Ucas) was defined as the amount of protease which produces an increment of one absorbance unit per min in the assay conditions [7].

2.3. Stability assays in aqueous–organic biphasic systems

Enzyme solutions of morrenain b II (lyophilized powder) and papain containing 1 mg/ml were prepared for stability assays in 30:70, 50:50 and 70:30 ratios of 0.1 M Tris–HCl buffer pH 8.5 and immiscible organic solvent. Each trial was performed by incubation of the mixture for 4 h at 40 °C, under magnetic stirring. The aqueous phase was sampled at different intervals and residual caseinolytic activity was quantified.

2.4. Enzymatic peptide synthesis

The condensation reaction was carried out in a 50:50 biphasic system, consisting of an aqueous phase (0.1 M Tris–HCl buffer pH 8.5), containing 0.08 mg/ml of enzyme (morrenain b II) and 12 mM Cys, and an organic phase (chloroform), containing 65 mM Phe.OMe and 12 mM Asp. The reaction was conducted at 40 °C in stoppered flask under magnetic stirring at 200 rpm. Aliquots (2 ml) of the reaction mixture were taken before starting and several times during the reaction period (40 h) and were mixed with 1 ml 5% (w/v) TCA to quench the reaction. TCA-treated samples were immediately centrifuged for 10 min at 2800 × *g* to separate the precipitate protein. After centrifugation, the organic phase was evaporated to dryness under reduced pressure while the aqueous phase was lyophilized. The samples were redissolved in acetonitrile and analyzed. Parallelly, blanks with identical composition but without the enzyme and with only the enzyme in a 50:50 biphasic system, consisting of 0.1 M Tris–HCl buffer pH 8.5 and chloroform, were carried out. Since morrenain b II belongs to the class of cysteine proteases, a comparison with papain under the same experimental conditions was also carried out.

2.5. Analytical control of peptide synthesis

To follow the course of the reaction, samples were analyzed by means of TLC, RP-HPLC, EI-MS and H-NMR.

2.5.1. TLC

A total of 100 ml of each sample in air dried silica gel G plates were placed in a ascending, saturated

chamber. The solvent system consisted of butanol: acetone:aqueous ammonia:water (10:10:5:2). Plates were spread with freshly prepared (0.2% (w/v)) ninhydrin in absolute ethanol solution containing 10% (v/v) acetic acid, and dried at 60 °C for 15 min.

2.5.2. RP-HPLC

Analyses were performed on a Merck–Hitachi System (Model L-6200 A) equipped with a C-18 Spherisorb ODS-2 (10 μm), 250 mm × 40 mm column, and the eluted material was monitored spectrophotometrically at 254 nm. The mobile phase consisted of 25% methanol and 0.075% perchloric acid. Aliquots of 50 μl were injected.

2.5.3. HPLC-MS

Reaction products were identified by HPLC-MS. Analyses were performed on VG-Quattro (Micromass Instruments S.A.), with a C-18 Nucleosil (120-5) (5 μm), 250 mm × 40 mm column (Scharlan). The technique of electrospray was used with positive ion reading (100–1000 uma). Nebulizer gas: N_2 (flow: 10 l/h). Drying gas: N_2 (flow: 450 l/h). Temperature of the source: 80 °C. Voltage of the capillary: 3.5 kV. Voltage focus: 55 V. Flow: 15 (ml/min). Elution: solvent A, H_2O containing 1% formic acid and solvent B, CH_3CN; A:B = 50:50. Volume of injection: 10–20 μl.

2.5.4. H-NMR

Analyses were performed on a Bruker equipment (200 MHz) connected to an analogical digitalis computational system (Aspect 3000). The samples were dissolved in D_2O at room temperature, until reaching a concentration above 10 mg.

3. Results and discussion

Although the presence of organic solvents may facilitate a catalytic process, as a rule, these solvents tend to cause disruption of hydrogen bonds and of hydrophobic interaction as well as changes in the dynamics and conformation of the protein [8,9]. With this in mind, biphasic systems were chosen for studying the ability of morrenain b II and papain to form peptide bonds, based on the stability performance of these enzymes in those media.

A statistical design by clustering 70 organic solvents according to their physical chemistry properties (descriptors), extracted from the literature, was carried out [10,11].

Ten immiscible organic solvents which were representative of each group, covering a wide range of π^* (solvent dipolarity/polarizability); α_1 (solvent hydrogen-bond acid or acidity); β_1 (solvent hydrogen-bond base or basicity); V_x (solvent volume); δ_H^2 (Hildebrand cohesive energy density) and $\log P$ (logarithm of the partition coefficient in a standard octanol–water two-phase system), were chosen for the morrenain b II and papain stability assays.

The comparison of the behavior of morrenain b II and papain in 30:70, 50:50 and 70:30 ratios of 0.1 M Tris–HCl buffer pH 8.5 and different immiscible organic solvents is shown in Figs. 1–4.

Papain stability profiles showed the highest caseinolytic activity retention (Ucas/mg of protein) in systems containing 30 or 50% (v/v) chloroform and 70% (v/v) ethyl ether, after 4 h of incubation at 40 °C (Figs. 1–3).

Likewise, morrenain b II showed the highest caseinolytic activity retention (Ucas/mg of protein) in the system containing 50% (v/v) chloroform, after 4 h of incubation at 40 °C (Fig. 4). Besides, morrenain b II showed remarkable stability in 50% (v/v) chloroform (69% of residual caseinolytic activity), after 40 h at 40 °C. It is important to point out that caseinolytic activity retention of morrenain b II in water was 8.2 (Ucas/mg of protein), after 4 h of incubation at 40 °C. This value was 70% lower than that obtained in a biphasic system containing 50% (v/v) chloroform.

Several authors have attempted to correlate and predict enzyme activity and/or stability in non-aqueous systems with certain parameters of the solvents such as dielectric constant, water solubility, Hildebrand solubility, three dimensional solubility parameter space and $\log P$. In practice, however, $\log P$ values are still used as a good guidance for purposes of solvent selection for biocatalysis [12–15]. However, since there is no straightforward correlation between $\log P$ values and the behavior of papain and morrenain b II in biphasic systems, it is necessary to carry out a more extensive analysis for understanding the effect of immiscible organic solvent on morrenain b II and papain.

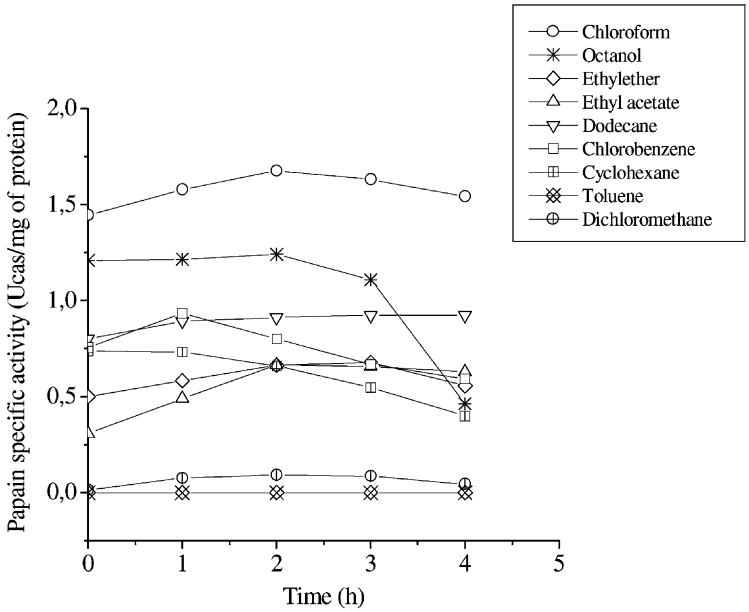

Fig. 1. Caseinolytic activity retention (Ucas/mg of protein) of papain, in a 30:70 Tris–HCl buffer pH 8.5 and different immiscible organic solvents, after 4 h of incubation at 40 °C.

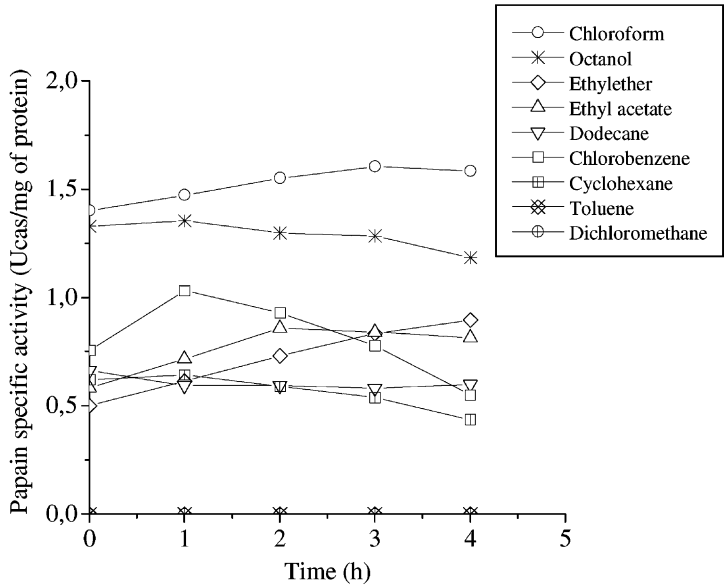

Fig. 2. Caseinolytic activity retention (Ucas/mg of protein) of papain, in a 50:50 Tris–HCl buffer pH 8.5 and different immiscible organic solvents, after 4 h of incubation at 40 °C.

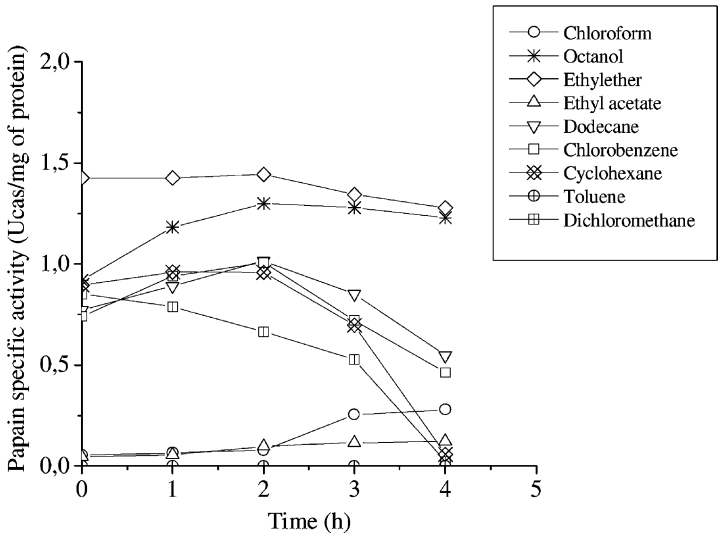

Fig. 3. Caseinolytic activity retention (Ucas/mg of protein) of papain, in a 70:30 Tris–HCl buffer pH 8.5 and different immiscible organic solvents, after 4 h of incubation at 40 °C.

On the basis of the obtained stability data, 50:50 Tris–HCl buffer pH 8.5 and chloroform were chosen as biphasic system for performing peptide synthesis.

The most common method for preparing enzymes to be used in non-aqueous solvents is lyophilization from a buffer at a suitable pH. The powders obtained can be directly added to an organic solvent containing a known amount of water [16].

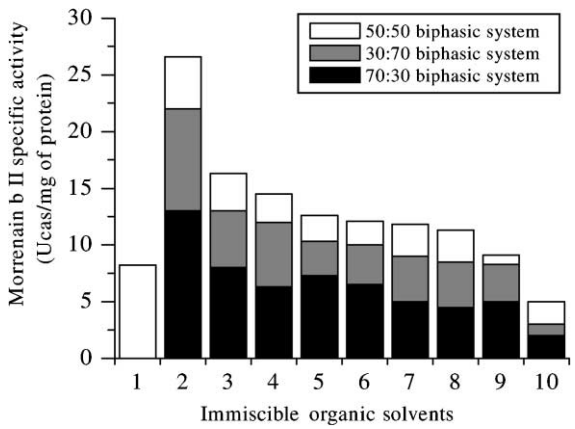

Fig. 4. Caseinolytic activity retention (Ucas/mg of protein) of morrenain b II, in a 30:70, 50:50 and 70:30 ratios of Tris–HCl buffer pH 8.5 and different immiscible organic solvents (2, chloroform; 3, chlorobenzene; 4, trichloroethylene; 5, cyclohexane; 6, ethylether; 7, toluene; 8, dichloromethane; 9, ethyl acetate; 10, octanol), after 4 h of incubation at 40 °C. Also 1 belongs to caseinolytic activity retention of morrenain b II in water, after 4 h at 40 °C.

The substrates were selected considering the availability of peptide commercial patterns, such as aspartame, that would facilitate the analysis of possible products of the synthesis reaction by RP-HPLC. Nevertheless, it was not possible to identify synthesis products by means of commercial patterns in RP-HPLC, since morrenain b II was not able to form Asp–Phe.OMe bonds.

Fig. 5 compares H-NMR spectra of the sample and blank (without the enzyme) and shows the disappearance of Phe.OMe (3.2–3.5 ppm) and the formation of at least one new compound (3.6 ppm). The reaction was followed by TLC and RP-HPLC. A typical RP-HPLC separation is shown in Fig. 6. Peak I was identified as Asp (2.86 min) by coelution with the isolated substrate, while Peak II belongs to the product of the synthesis reaction. The TLC analysis is shown in Fig. 7. In this figure, two spots are seen as product of the synthesis reaction because it was unpurified with Phe.OMe, while Cys probably contained some cystine.

Mass spectrometry (EI-MS) analysis of the synthesis product revealed ion masses of 255.2 and 341.5 and 385.5 (Fig. 8), which corresponded to the peptide cystine–Phe.OMe.

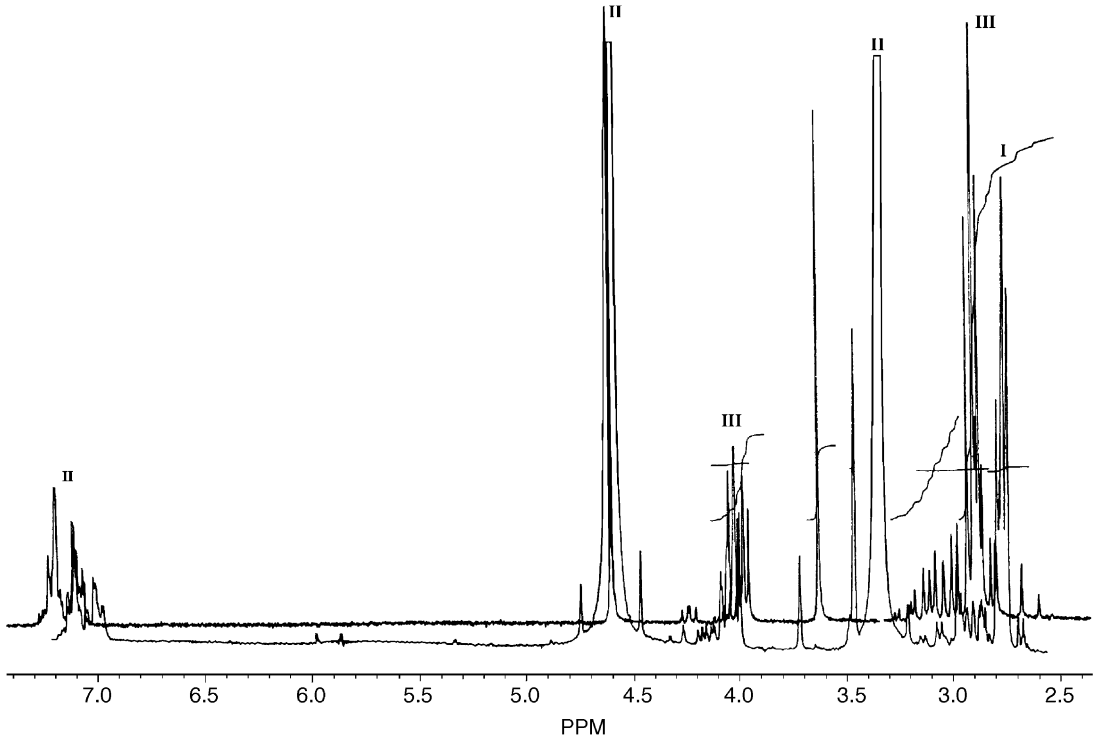

Fig. 5. H-NMR of the reaction mixture (upper line) and its respective blank (lower line) after 1 h at 40 °C and 200 rpm. The labeled peaks are as follows: I (Asp); II (Phe.OMe) and III (Cys). The reaction mixture was 50:50 biphasic system, consisting of 0.08 μg/ml of morrenain b II and 12 mM Cys dissolved in 0.1 M Tris–HCl buffer pH 8.5 and 65 mM Phe.OMe and 12 mM Asp in chloroform.

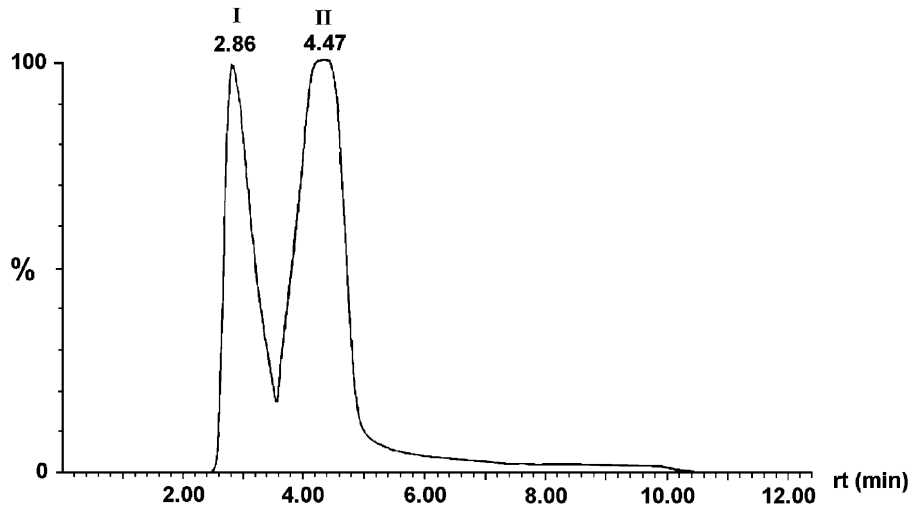

Fig. 6. RP-HPLC of the reaction mixture after 1 h at 40 °C and 200 rpm. The labeled peaks are as follows: I (Asp) and II (product of the synthesis). The reaction mixture was 50:50 biphasic system, consisting of 0.08 μg/ml of morrenain b II and 12 mM Cys dissolved in 0.1 M Tris–HCl buffer pH 8.5 and 65 mM Phe.OMe and 12 mM Asp in chloroform.

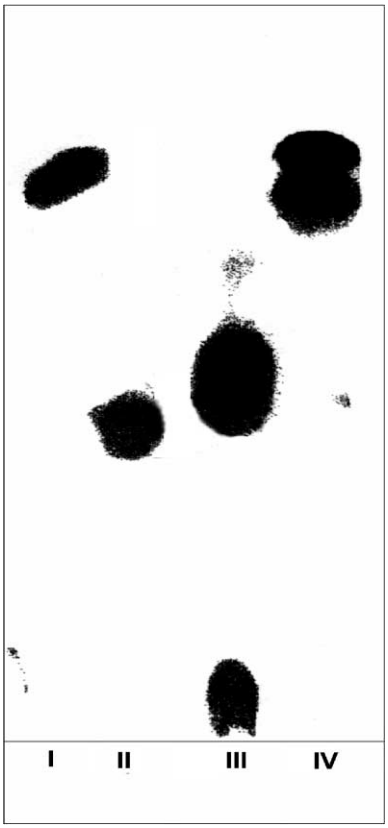

Fig. 7. TLC of the I (Phe.OMe); II (Asp); III (Cys) and IV (product of the synthesis after 1 h of reaction at 40 °C and 200 rpm). The reaction mixture was 50:50 biphasic system, consisting of 0.08 μg/ml of morrenain b II and 12 mM Cys dissolved in 0.1 M Tris–HCl buffer pH 8.5 and 65 mM Phe.OMe and 12 mM Asp in chloroform.

Although Cys was added only as activator of morrenain b II, it also acted as acyl donor substrate in the studied synthesis reaction. Cystine is the name of the Cys dimmer and its disulfer bridge can be formed under oxidizing conditions and split by the action of reducing agents (Fig. 9). When some morrenain b II gratuitous inducers, such as sodium sulfite and 2-mercaptoethanol, replaced Cys the enzyme did not catalyze the synthesis of any products, showing the high specificity of morrenain b II in the formation of cystine–Phe.OMe peptide.

A more conclusive evidence of the identity of the compound formed was obtained from a series of experiments in which the three substrates (Cys, Asp and Phe.OMe) were incubated with the enzyme as single compounds and in all possible combinations. The product cystine–Phe.OMe was formed in those combinations in which Cys and Phe.OMe were present.

Although the course of the reaction was followed by means of RP-HPLC for 40 h, within the first 30 min, the concentration of substrate Phe.OMe steadily decreased, accompanied by a rise in the concentration of cystine–Phe.OMe, which reached its maximum concentration after 1 h. No other products were formed in the remaining time. Besides, the concentration of the synthetic reaction product was not significantly enhanced by molar excess up to 325 mM of the acyl donor amino acid.

No other compounds of synthesis incapable of absorbing in UV were detected by HPLC analysis with IR detector, which indicates that the enzyme was incapable of synthesizing Asp–Asp or Cys–Cys bonds.

After consuming the ester substrate, the concentration of the synthesis product remained unchanged, indicating that the peptide formed was not hydrolyzed under the chosen reaction conditions. This observation supports the idea that the peptidase activity of morrenain b II was negligible while esterase activity was significant.

On the other hand, only amino acids with unprotonated α-amino groups take part in the coupling reaction. Then, the effective nucleophile concentration is determined by the p*K*a of the α-amino groups, being about pH 9 for the common amino acids at 40 °C [17]. Thus, pH values above 9 might have been optimum in this respect. However, under such conditions, morrenain b II drastically decreased the overall activity [18].

Our results demonstrated that at pH 8.5 there was an effective nucleophile concentration adequate for attacking the carbonyl carbon of the acyl-enzyme complex previously formed, and for obtaining a successful coupling of the amino acid ester.

A more hydrophobic character of the nucleophilic amino acid residue might facilitate its incorporation into a peptide bonds [19].

Unlike morrenain b II, papain was unable to synthetize cystine–Phe.OMe peptide. These results are in agreement with those reported by Mitin et al. [20], who successfully synthesized a number of di-, tri-, tetra-, and pentapeptides using papain as catalyst, at alkaline pH values. The best results were obtained using

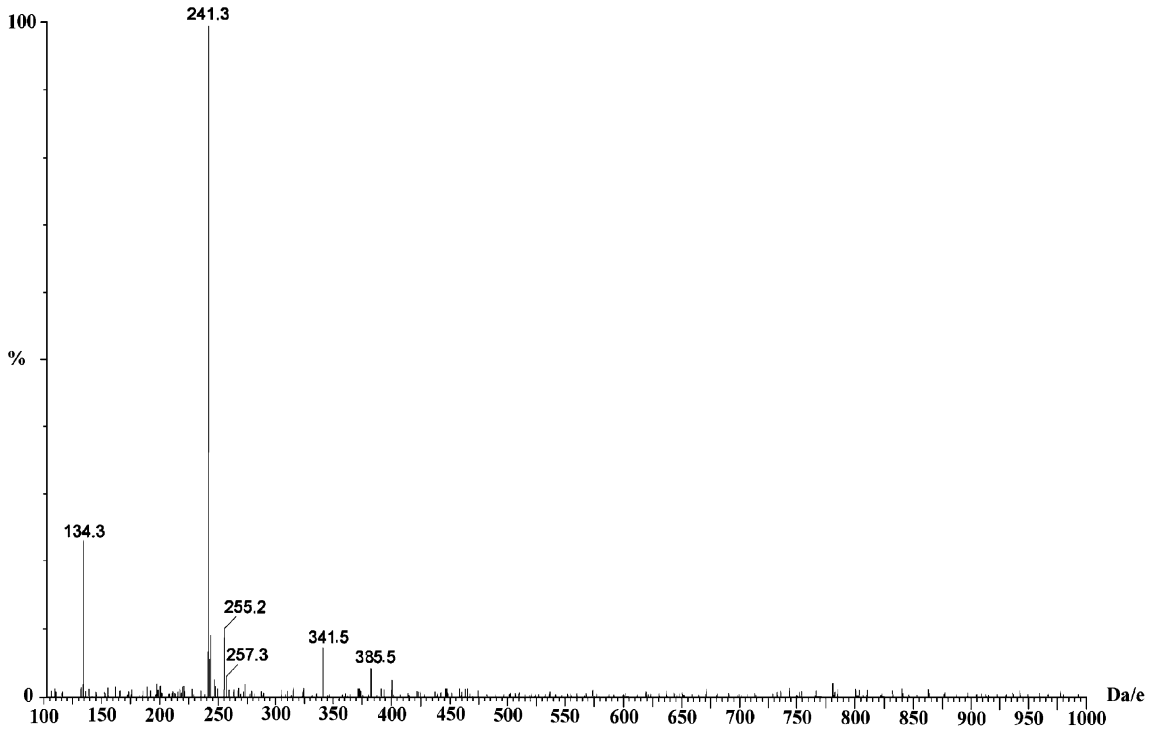

Fig. 8. Mass spectrum of material eluted of RP-HPLC. Ion masses (*M*/*Z*) of 255.2, 341.5 and 385.5 correspond to the peptide cystine–Phe.OMe; Ion masses (*M*/*Z*) of 134.3 and 241.3 are Asp and cystine, respectively. Condensation reaction carried out in a 50:50 biphasic system, consisting of 0.08 μg/ml of morrenain b II and 12 mM Cys in 0.1 M Tris–HCl buffer pH 8.5 and 65 mM Phe.OMe and 12 mM Asp in chloroform.

Z-aminoacid methyl esters and amino acid amides, whereas free amino acids were claimed not to be suitable as amino acids components [20]. These reactions of kinetically controlled peptide synthesis, catalyzed by papain, were the first described in the literature.

Although in this work morrenain b II did not catalyze the formation of the predicted Asp–Phe.OMe bonds, the described preliminary results indicate that protease could be used for the production of the peptide cystine–Phe.OMe.

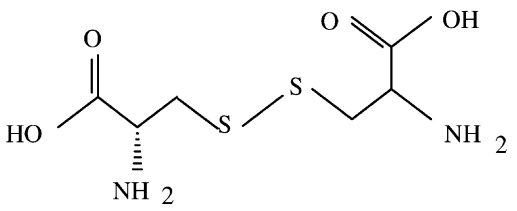

Fig. 9. Chemical structure of cystine.

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