World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:5, No:4, 2011

Synthesis of Peptide Amides using Sol-Gel Immobilized Alcalase in Batch and Continuous Reaction System

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Abstract**—**Two commercial proteases from *Bacillus licheniformis* (Alcalase 2.4 L FG and Alcalase 2.5 L, Type DX) were screened for the production of Z-Ala-Phe-NH2 in batch reaction. Alcalase 2.4 L FG was the most efficient enzyme for the C-terminal amidation of Z-Ala-Phe-OMe using ammonium carbamate as ammonium source. Immobilization of protease has been achieved by the sol-gel method, using dimethyldimethoxysilane (DMDMOS) and tetramethoxysilane (TMOS) as precursors (unpublished results). In batch production, about 95% of Z-Ala-Phe-NH_2 was obtained at 30°C after 24 hours of incubation. Reproducibility of different batches of commercial Alcalase 2.4 L FG preparations was also investigated by evaluating the amidation activity and the entrapment yields in the case of immobilization. A packed-bed reactor (0.68 cm ID, 15.0 cm long) was operated successfully for the continuous synthesis of peptide amides. The immobilized enzyme retained the initial activity over 10 cycles of repeated use in continuous reactor at ambient temperature. At 0.75 mL/min flow rate of the substrate mixture, the total conversion of Z-Ala-Phe-OMe was achieved after 5 hours of substrate recycling. The product contained about 90% peptide amide and 10% hydrolysis byproduct.

Keywords**—**packed-bed reactor, peptide amide, protease, sol-gel immobilization.

I. Introduction

THE majority of known bioactive peptides possess a C-terminal amide moiety. Structure-activity studies established that the C-terminal amide is very important to their activity [1]. Several bioactive peptides possess functions covering a broad range of activities. It was observed that antimicrobial peptides are bactericidal, but in the meantime can also possess antiviral, antifungal, antitumor, and immunomodulatory activities [2]. The operational stability of a biocatalyst is usually critical in terms of its acceptance for an industrial biotransformation. This applies whether it is used in a continuous process or reused repeatedly in batch.

Proteases represent a class of enzymes of emerging interest, used as catalysts for the cleavage of proteins [3], [4] and synthesis and modification of peptides [5]. For such reactions it is often desirable to use organic media, so the operational stability of proteases under these conditions is important. To improve the enzyme stability in organic media, various methods of immobilization have been developed, such as physical adsorption of enzymes on a solid support [6], encapsulating techniques using sol-gel method [7], covalent binding through the appropriate groups [8], [9]. The immobilization of proteases on a solid support can offer several advantages over the free enzyme, including easy handling, recovery from the reaction medium, reuse and/or operation in continuous reactors [10].

Sol-gels are a new class of materials that have been found to be suitable for the immobilization of enzymes and other biological molecules. Encapsulation of enzymes within sol-gel matrices could be more efficient compared to the other immobilization methods, considering the entrapment of larger amount of enzyme, thermal and chemical stability, simplicity of preparation without any covalent modification, and control of pore size [5].

In this study, we tested Alcalase 2.4 L FG protease to catalyze the ammonolysis of Z-Ala-Phe-OMe, using ammonium carbamate as ammonium source. Enzyme entrapment in sol-gel matrices represents a mild technique, because the polymers are not toxic, and can even be used to entrap living cells. By using this immobilization method, it was intended to supply the enzyme with sufficient operational stability for continuous exploitation. The versatility of the obtained biocatalyst was investigated in the batch process, followed by utilization of sol-gel immobilized Alcalase 2.4 L FG in a packed-bed reactor, to obtain a continuous production of peptide amides.

II. Materials and Methods

A. Materials

Alcalase 2.4 L FG (batch PLN05354 and PLN05361) and Alcalase 2.5 L, Type DX were obtained from Novozyme and used as purchased. Silane precursors tetramethoxysilane (TMOS, 98%) and dimethyldimethoxysilane (DMDMOS, 96%) were products of Brunschwig Chemie (The Netherlands), and Fluka, respectively. The C-terminal methyl ester of dipeptide, Z-Ala-Phe-OMe (HPLC purity >98 %) was purchased from Bachem AG, Switzerland. The C-terminal *tert*-butyl ester of dipeptide, Z-Ala-Phe-O^{t}Bu, and the reference peptides Z-Ala-Phe-NH_2, Z-Ala-Phe-OH and Boc-Gly-Phe-Phe-Leu-O^{t}Bu were provided by MSD, The Netherlands. The solvents were stored over 4-Å molecular

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sieves and used without further purification. All other common chemicals used were of analytical grade.

B. Preparation of Alcalase-Immobilized in Sol-Gel Matrices derived from DMDMOS and TMOS

The immobilization method [11] based on the Reetz procedure for the entrapment of lipases [7] has been used. Alcalase 2.4L FG solution batch PLN05354 (58 mg protein/mL) was used for immobilization. In a 10 mL glass vial, Alcalase solution (3.12 mL), PEG 20000 (0.8 mL), 1M NaF (0.4 mL), and isopropyl alcohol (0.8 mL) were mixed (magnetic stirring, 600 rpm). By continuous stirring, 24 mmoles silane precursors (DMDMOS/TMOS, molar ratio 1:1) were added. The resulting mixture was vigorously stirred at ambient temperature until the gel formation started. The gel was kept for 24 hours at 4ºC in the refrigerator to complete polymerization. The bulk gel was washed with milli Q water (40 mL), isopropyl alcohol (20 mL) and n-hexane (20 mL) and dried at ambient temperature for 48 hours. Finally, it was crushed in a mortar and kept in the refrigerator. The washing solutions were analyzed for protein content, and the activity of immobilized enzyme was determined using the model reaction (Fig 1).

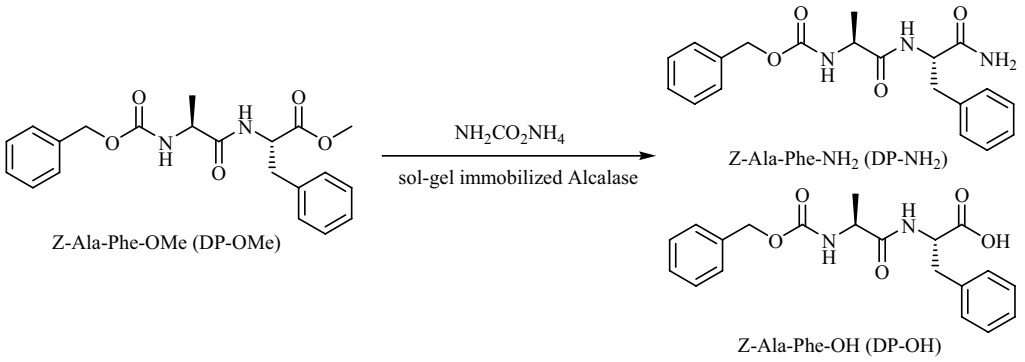

Fig 1 Reaction scheme of enzymatic synthesis of the model dipeptide amide (DP-NH_2, dipeptide amide; DP-OH, dipeptide free acid; DP-OMe, dipeptide methyl ester; Z, benzyoxy-carbonyl protecting group)

C. Enzymatic Ammonolysis of Z-Ala-Phe-OMe in Batch System

The best reaction conditions for maximum amide yield and minimum secondary hydrolysis amount were determined previously [12], [18]. In the batch system, the reaction of model dipeptide was performed in a 24-tubes carrousel (GreenHouse PlusTM Parallel Synthesizer; Radleys Discovery Technologies) with controlled temperature, cooling system and magnetic stirring. The test tubes were charged with a mixture of Z-Ala-Phe-OMe substrate (10 mM), ammonium carbamate (1:10 molar ratio) and 5 mL of a solvent mixture composed of dimethylformamide (DMF) and *tert*-butanol (^{t}BuOH), at 17.5:82.5 ratio (v/v). The mixture was thermostated at 30°C for 30 minutes. The reaction was initiated by the addition of 10 µL of Alcalase solution (the final concentration of protein in the reaction mixture was 0.02 mg/mL) or 2.5 mg/mL of sol-gel immobilized protease. Samples taken at different time intervals were diluted by addition of an equal volume of acetonitrile, and subsequently analyzed by RP-HPLC. The components were identified using the appropriate reference compounds. The relative percentage yields (molar conversion) were calculated from peak area integration (1 and 2, where $A_{DP\text{-}NH2}$, $A_{DP\text{-}OH}$ and $A_{DP\text{-}OMe}$ represent the peak area of dipeptide amide, dipeptide free acid, and dipeptide methyl ester, respectively). Amidation specific activities were expressed as the amount of formed Z-Ala-Phe-NH_2 (in micromole) in 1 minute interval by 1 mg of protein. The reaction without enzyme did not give any product in the same condition. All reactions were performed in duplicate.

D. Enzymatic Ammonolysis of Z-Ala-Phe-OMe in a Continuous System

In the continuous system, the substrate mixture (50 mM) was homogenized at 300 rpm on a magnetic stirrer, and introduced into the column reactor with a peristaltic pump. The reaction was carried out at ambient temperature. The immobilized Alcalase 2.4L FG (1.2 g) and ammonium carbamate (3.0 g) were packed layer by layer (Fig. 2) in a glass column (0.68 cm ID, 15.0 cm long). The well mixed substrate mixture was introduced at the top of the column, at a flow rate of 0.15 or 0.75 mL/min. The product was removed at the bottom of the column (Fig. 3). After each run the column was washed with 10 mL of solvent mixture DMF/^{t}BuOH (17.5:82.5, v/v). The relative percentage yield (molar conversion) was calculated from the HPLC analysis data.

E. Hydrolysis Activity Assay

The hydrolysis of tert-butyl esters of peptides (Z-Ala-Phe-O^{t}Bu and Boc-Gly-Phe-Phe-Leu-O^{t}Bu) was performed in a 24-tubes carrousel (GreenHouse PlusTM Parallel Synthesizer; Radleys Discovery Technologies) with controlled temperature, cooling system and magnetic stirring. The test tubes were charged with a mixture of peptide substrate (10 mM), and 1 mL of a solvent mixture composed of DMF and

phosphate buffer 10 mM, pH 7.0 of the composition 50:50 (v/v) as published previously [13], [14]. The mixture was kept for 30 minutes at 40°C. The reaction was initiated by the addition of 50 μL of Alcalase solution (the final concentration of protein in the reaction mixture was 0.02 mg/mL). Samples taken at different times during the course of the reaction were diluted by the addition of an equal volume of acetonitrile and analyzed by RP-HPLC. The identity of the compounds was determined with the use of reference compounds. Hydrolysis specific activities were expressed as the amount of formed free peptide acid (in micromole) in 1 minute interval by 1 mg of protein. All the reactions were performed in duplicate.

Fig. 2 Column reactor filled with sol-gel immobilized Alcalase and ammonium carbamate

F. Protein Content Determination

The Bradford method [15] was used to measure the protein concentration of solutions with dissolved protein at 595 nm with a SAFIRE spectrophotometer (Tecan Benelux BVBA, Giessen, the Netherlands), using bovine serum albumin (BSA) as a standard.

G.HPLC Analysis

The ammonolysis and hydrolysis reaction mixtures were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC) on an Atlantis T3 3 μm, 2.1 mm x 100 mm column (Waters) thermostated at a temperature of 30°C using a HPLC system (Waters) equipped with UV dual wavelength detector and autosampler. The eluting components were detected at 220 nm. The mobile phase A was 0.1% trifluoroacetic acid in water, while mobile phase B was 0.1% trifluoroacetic acid in acetonitrile. The mobile phase flow rate was 0.5 mL/min. A linear gradient from 2 to 98% acetonitrile was applied from 0 to 17.5 min. From 17.5 to 22 min, the mobile phase composition was constant 98% acetonitrile and 2% water.

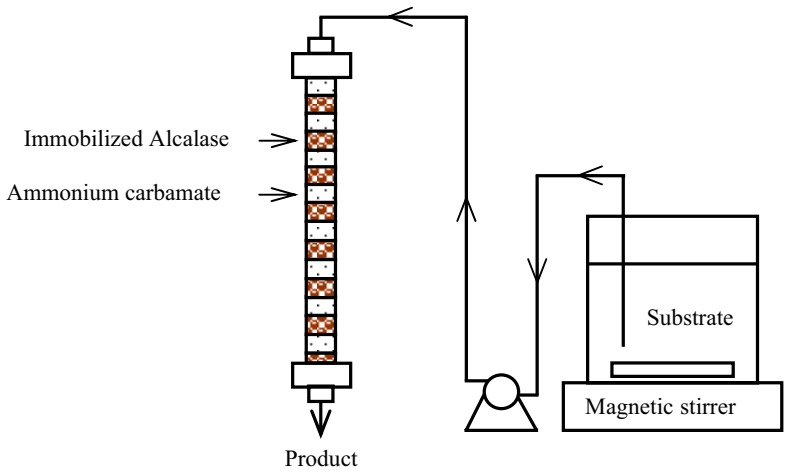

Fig. 3 Schematic diagram for continuous ammonolysis of Z-Ala-Phe-OMe by immobilized Alcalase at ambient temperature

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

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

H. Electrophoresis Assay

Acrylamide gel electrophoresis was performed using the XC II™ mini-cell electrophoresis system (Novex) and precast 10% Bis–Tris NuPAGE gels according to the manufacturer's protocols. The proteins were visualized by staining the gels with Coomassie Blue dye or by silver staining [16].

III. Results and Discussion

A. Enzyme Selection

Enzymatic synthesis of amidated peptides by ammonolysis of peptide metyl esters and hydrolysis of *tert*-butyl esters was investigated (Fig. 1). The aim of this study was to select the most efficient protease for peptide amidation and/or C-terminus deprotection. Two commercial proteases, produced by the same company (Novozyme), were tested for their activity in the amidation and hydrolysis of C-terminal peptide esters. Results are shown in Fig. 4. It can be observed that Alcalase 2.4L FG is more active for the ammonolysis of Z-Ala-Phe-OMe (specific activity: 13 μmole min^{-1} mg $protein^{-1}$) than Alcalase 2.5L, Type DX (specific activity: 11.1 μmole min^{-1} mg $protein^{-1}$). In order to investigate the substrate specificity of proteases, the model dipeptide (Z-Ala-Phe-O′Bu) and a tetrapeptide (Boc-Gly-Phe-Phe-Leu-O′Bu) were used. Although the activities were lower for the longer peptide, no significant differences were detected between the two Alcalase enzymes concerning the activity values for hydrolysis of *tert*-butyl esters of the di- and tetrapeptide. Therefore, protease Alcalase 2.4L FG was selected for the subsequent experiments.

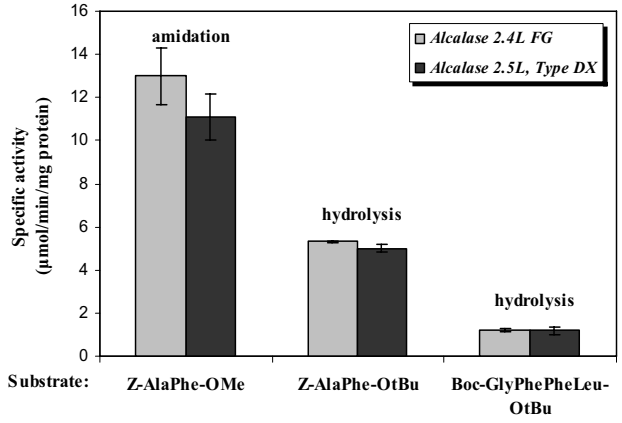

Fig. 4 Screening of proteases for the ammonolysis and hydrolysis of peptides

B. Enzyme Characterization by SDS-PAGE Electrophoresis

Since Alcalase 2.4L FG and Alcalase 2.5L DX are commercial crude preparations produced by *Bacillus licheniformis* strain, with the main enzyme being Subtilisin A (an alkaline serine protease), it was important to study their purity by SDS-PAGE electrophoresis. Both enzyme preparations presented several protein bands up to ca. 55 kDa, the most intense at 29 kDa corresponding to Subtilisin A (Fig. 5). It must be pointed out that the Alcalase 2.4L FG preparation contained protein bands between 55 and 29 kDa that are not presented in the composition of Alcalase 2.5L DX. There were also several protein bands at lower molecular weight (below ca. 22 kDa), indicating that the enzyme preparations had a high content of protein contamination, probably autolysis products of the enzymes.

C. Reproducibility of Commercial Alcalase 2.4L FG Batches

Different batches of enzymes may vary in quality and each new batch of enzyme should be tested prior to routine use. Therefore, the variations in the composition and properties of the batches of crude Alcalase used for immobilization, and the reproducibility of enzyme immobilization via sol-gel entrapment has been investigated. Different Alcalase batches were characterized and tested for the amidation of Z-Ala-Phe-OMe.

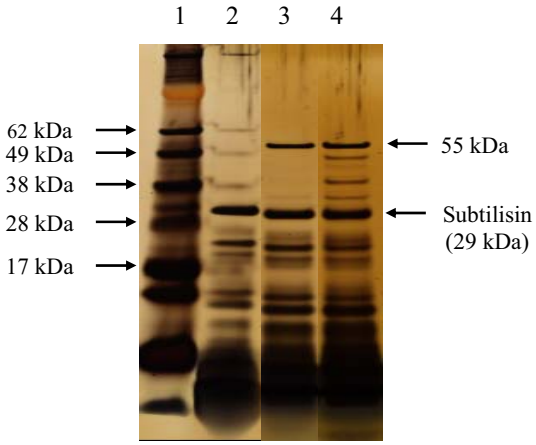

Fig. 5 SDS-polyacrylamide gel electrophoresis of proteases. Lane 1, standard molecular weight markers; lane 2, pure Subtilisin A protease; lane 3, Alcalase 2.5L. Type DX; lane 4, Alcalase 2.4L FG batch PLN05354

1. Protein Composition Reproducibility

To obtain a qualitative indication of the protein purity of the enzyme and the variation in protein composition, an SDS-polyacrylamide gel electrophoresis (PAGE) using Coomassie blue staining of different batches of Alcalase 2.4L FG was performed (Fig. 6). It was observed that the two batches displayed a large band at approximately 29 kDa, which corresponded to the molecular weight of Subtilisin. Differences in the SDS-PAGE were not observed between the Alcalase batches, revealing a good reproducibility in protein composition.

2. Soluble Proteases Activity Assay

The reproducibility of catalytic activity of different soluble Alcalase batches was investigated in the amidation of Z-Ala-Phe-OMe. The protein concentration of commercial Alcalase preparations was determined by Bradford method. A small

variation of protein concentration values was observed, with 6.93% standard deviation (Table I). The enzyme activities were calculated in the first 5 minutes of the reaction. The reproducibility of activity values of the two Alcalase batches was good, with a standard deviation of only 2.65%.

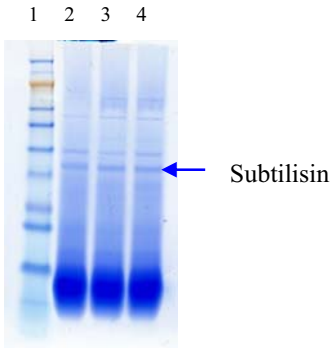

Fig. 6 SDS-polyacrylamide gel electrophoresis of the different batches of alcalase. Lane 1, standard molecular weight markers; lane 2 and 3, Alcalase 2.4L FG batch PLN05354 (the same batches in a different experiment); lane 4, Alcalase 2.4L FG batch PLN05361

3. *Immobilized Proteases Activity Assay*

The Alcalase batches were immobilized twice under the same conditions, and tested for reproducibility of activity in the model reaction. The silane precursors used for immobilization were dimethyldimethoxysilane (DMDMOS) and tetramethoxysilane (TMOS) at a molar ratio of 1:1. As evident from Table II, the percentage of peptide amide in the final product, as well as the specific activity in the initial stage of the reaction showed excellent reproducibility for the two batches. Determination of the protein content in the water washing solutions revealed that between 70 and 73% of the enzyme has been immobilized, and the degree of immobilization had a standard deviation of 1.53%. Using these preparations, total conversion of the substrate peptide ester was achieved within 24 hours, and the product contained

TABLE I
REPRODUCIBILITY OF DIFFERENT BATCHES OF COMMERCIAL ALCALASE PREPARATIONS, IN RELATION TO PROTEIN CONTENT AND CATALYTIC ACTIVITY IN THE AMIDATION OF Z-ALA-PHE-OME

Alcalase batch no. PLN	Protein concentration (mg/ml) ± SD %	Activity (μmol min^{-1} mg $protein^{-1}$) ± SD %
[a] 05354 [b] 05354 05361	50 ± 6.93	14.8 ± 2.65

[a, b] The same batches in a different experiment units

about 95% of the desired amide, Z-Ala-Phe-NH_2. The percentage of peptide amide in the final product, as well as the total activity in the initial stage of the reaction, showed excellent reproducibility as well, with a standard deviation of 0.11% and 0.15%, respectively.

TABLE II
REPRODUCIBILITY OF COMMERCIAL ALCALASE BATCHES

Alcalase batch no. PLN	Entrapment yield ± SD %	Conversion after 24 hours			Total activity (μmol h^{-1} mg gel^{-1})
		DP-NH_2 ± SD %	DP-OH ± SD %	DP-OMe ± SD %	
[a] 05354 [b] 05354 05361	71.7 ± 1.53	95.4 ± 0.11	4.6 ± 0.11	0.0 ± 0.0	3.81 ± 0.15

[a, b] The same batches in a different experiment units

D.Synthesis of Z-Ala-Phe-NH_2 in a Packed-Bed Immobilized Enzyme Reactor

Immobilization is considered the most important method to improve the stability of enzymes, as much as they are destined for possible industrial applications. Reusability is a crucial feature in practical applications of biocatalysts. Inactivation is the most prominent drawback for large-scale use of immobilized enzymes. Since packed-bed reactors are continuing to dominate the large-scale industrial applications of immobilized enzymes, we investigated such a reaction system for the amidation of peptide esters. Continuous production of Z-Ala-Phe-NH_2 with immobilized Alcalase from *Bacillus licheniformis* was studied. The operational stability of protease entrapped in sol-gel matrices was examined for repeated use in a continuous enzyme reactor system for the synthesis of Z-Ala-Phe-NH_2, at ambient temperature (Fig. 7). After each first run of substrate solution through the column, at a flow rate of 0.15 mL/min, about 60% of peptide amide yield was achieved. As evident from Fig. 7, the immobilized biocatalyst showed remarkable operational stability, as the relative catalytic efficiencies (related to the yield of amide product measured for the first run of substrate solution through the column) were above 0.98 for the amidation catalyzed by sol-gel entrapped protease, even after 10 cycles of repeated use. After 5 hours of substrate recycling, at a flow rate of 0.75 mL/min, a total conversion of Z-Ala-Phe-OMe was achieved (Fig. 8). The product mixture contained about 90% of peptide amide and 10% of hydrolysis byproduct. The reusability of immobilized protease is essential for cost-effective use of the enzyme in a continuous process [17].

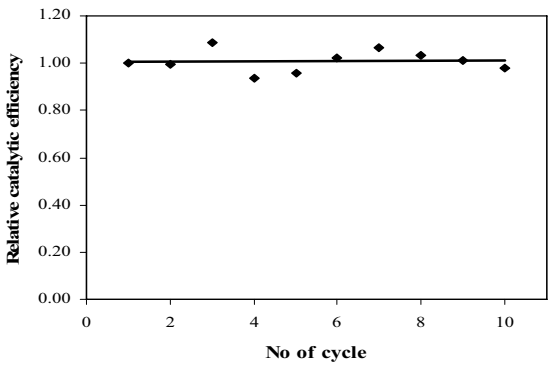

Fig. 7 Reusability of silica-immobilized Alcalase in a continuous process

This first set of preliminary experiments clearly demonstrates the efficiency and stability of sol-gel entrapped protease as biocatalyst for the synthesis of dipeptide amides in continuous reaction system. Further studies concerning optimization of reaction parameters of this process (substrate solution flow rate, temperature, enzyme/substrate ratio), as well as half-life of immobilized protease and long-term continuous operating in the reactor, are currently being investigated.

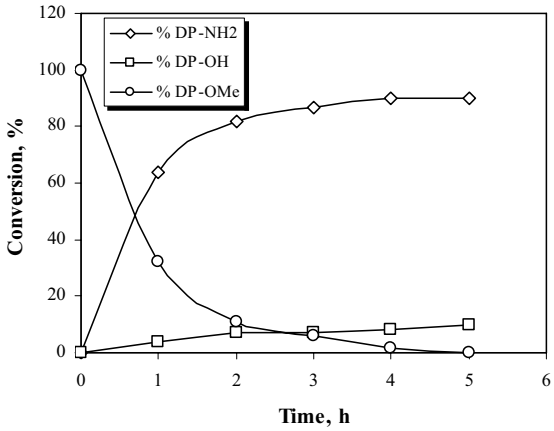

Fig. 8 Time-course production of Z-Ala-Phe-NH_2 in the immobilized Alcalase-catalyzed amidation of Z-Ala-Phe-OMe

IV. Conclusions

The obtained results highlight the suitability of sol-gel immobilized Alcalase to serve as a biocatalyst in the modification of peptides. Alcalase 2.4 L FG (*Bacillus licheniformis*) immobilized by encapsulation in sol-gel matrices derived from DMDMOS and TMOS at a molar ratio of 1:1 was suitable for Z-Ala-Phe-NH_2 production, using ammonium carbamate as ammonium source. In addition, it could be used for continuous operation in a packed-bed reactor system showing good operational and mechanical stability.

Acknowledgment

The work of L. N. Corîci was partially supported by the strategic grants POSDRU 6/1.5/S/13, 2008 of the Ministry of Labour, Family and Social Protection, Romania, co-financed by the European Social Fund-Investing in People.

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