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High isolated yields in thermolysin-catalysed synthesis of *Z*-L-aspartyl-L-phenylalanine methyl ester in toluene at controlled water activity

Luigi De Martin, Cynthia Ebert, Lucia Gardossi* and Paolo Linda

Dipartimento di Scienze Farmaceutiche, Università degli Studi, Piazzale Europa 1, 34127 Trieste, Italy

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Abstract—*Z*-L-Aspartyl-L-phenylalanine methyl and ethyl esters were synthesised enzymatically in toluene by means of the zinc protease thermolysin adsorbed onto Celite R-640®, which is a porous support able to control the hydration of the protein. The conversion of the two derivatised amino acids into the desired products was complete, leading to >90% isolated yields. Moreover, working with equimolar concentrations of the reactants no purification steps were required. Thermolysin adsorbed onto Celite R-640® is shown to be a practical tool to synthesise biologically active peptides in organic media.

The benefits of using enzymes in organic synthesis and, in particular, for the preparation of optically pure drugs are well recognised.[1] In the last few years much attention has been paid to protease-catalysed peptide synthesis by using the reverse of the hydrolytic reaction, namely thermodynamically controlled synthesis.[1] The enzymatic approach offers some advantages over chemical methods since enzymatic reactions are often performed under mild conditions and without protection of the side chains of the amino acid derivatives.[2] Aspartame (α-L-Asp-L-Phe-OMe) is one of the best known peptides obtained through enzymatic synthesis. This dipeptide is an artificial sweetener which is 200 times sweeter than sucrose and used throughout most of the world.[3]

The precursor of aspartame (X-L-Asp-L-Phe-OMe, where X is an *N*-protecting group) has been synthesised by several research groups starting from *N*-protected L-aspartic acid (X-L-Asp) and L-phenylalanine methyl ester (L-PheOMe) using the zinc protease thermolysin.[4–7]

The first approach reported in the literature included the thermodynamically controlled synthesis of the precursor of aspartame in water.[5,8] Since in water the equilibrium constant is shifted towards the hydrolysis products ($K_{eq}=1.5\ M^{-1}$), unsatisfactory yields were obtained in the dilute aqueous medium.[3] However, over the last few years Halling and co-workers have obtained very high yields in the thermolysin-catalysed synthesis of *Z*-L-Gln-L-Leu-NH_2 and *Z*-L-Asp-L-Phe-OMe using aqueous suspensions of the substrates.[9–11]

An alternative approach could be represented by the partial substitution of the aqueous medium with an organic solvent which can shift the thermodynamic equilibrium towards synthesis.[12] Therefore, reactions have been performed in either water/organic solvent mixtures[13,14] or in two phase systems.[6,15–17]

In principle, the use of pure organic solvents in enzymatic synthesis should be beneficial for the equilibrium shift, for the recovery of the product and the recycling of the catalyst.[18]

Recently we reported that thermolysin is very active in hydrophobic organic solvents when the hydration of the protein is controlled by adsorbing the enzyme onto Celite R-640® rods.[19]

In the present paper we present the thermolysin-catalysed synthesis of *Z*-L-aspartyl-L-phenylalanine methyl and ethyl esters in a hydrophobic solvent at controlled water activity (a_w), using equimolar concentrations of the substrates.

Keywords: aspartame; peptide synthesis; thermolysin; organic solvent; thermodynamically controlled synthesis; water activity.

* Corresponding author. Fax: +39-040-52572; e-mail: linda@univ.trieste.it

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Table 1. Equilibrium conversions in the synthesis of *Z*-L-Asp-L-Phe-OR (R = Me, Et) catalysed by thermolysin adsorbed onto Celite R-640® rods in toluene at controlled a_w[a]

Acylating agent[b]	Nucleophile[b]	a_w (t_0)	a_w (equilibrium)	Conversion (%)[d]	Time (h)
Z-L-Asp	L-Phe-OEt	0.78	0.81	96	220
Z-L-Asp	L-Phe-OMe	0.73	0.76	99	240
Z-L-Asp	L-Phe-OMe[c]	0.77	0.80	98	144

[a] Experimental conditions: reaction volume: 1 mL of toluene, 30°C, 5 mg/mL of enzyme adsorbed onto 200 mg of Celite R-640® rods.
[b] 80 μmol suspended in 1 mL of toluene.
[c] 160 μmol suspended in 1 mL of toluene.
[d] Final conversions were evaluated both by RP-HPLC and by titration of *Z*-L-Asp-CO_2H with 0.01 M NaOH. Products were characterised by ^{1}H NMR and ES–MS.

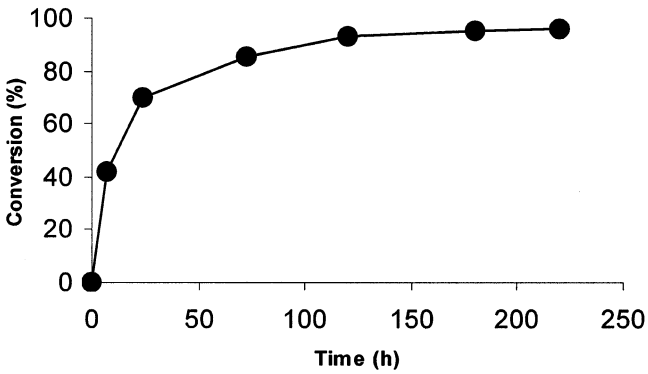

Figure 1. Time course of thermolysin-catalysed *Z*-L-Asp-L-Phe-OEt synthesis in toluene at controlled a_w.

Enzymatic synthesis of *Z*-L-Asp-L-Phe-OR

Thermolysin was adsorbed onto Celite R-640® directly in toluene, equilibrated for 24 hours at 30°C in a thermostatted orbital shaker and gave at equilibrium, an a_w value between 0.73 and 0.78. Enzymatic reactions were carried out simply by adding the substrates into the same vial used for the adsorption and storage of the enzyme.[†] It must be underlined that the substrates were only partially soluble in toluene, so that the reaction was performed in an organic solvent suspension of the two substrates.

The equilibrium conversions for the synthesis of *Z*-L-Asp-L-Phe-OR dipeptides are reported in Table 1.

The a_w values measured at the equilibrium of the reaction were close to those measured before the start of the reaction although water is one of the reaction products. The absorption properties of Celite R-640® made it possible to shift the equilibrium of the reaction towards synthesis obtaining quantitative conversions, even when equimolar amounts of non-activated substrates were employed.

[†] Experimental conditions: 1 mL of toluene, 5 mg of thermolysin adsorbed onto Celite R-640® rods following a procedure previously reported,[19] T = 30°, 80 μmol of *Z*-L-Asp, 80, 160 μmol of L-Phe-OR·HCl, 100 and 180 μmol $Na_2CO_3 \cdot 10H_2O$ were used to obtain the free amine[20] directly in the reaction medium.

It is widely documented that the removal of the product, either by precipitation[5,19] or by extraction[16,17] from the reaction medium, can be the driving force for the reaction to reach high conversions in short times. In the present cases, the product accumulated exclusively in the liquid phase, and no precipitation was detected during the reaction course. A typical time course of the reaction is reported in Fig. 1.

As shown in Fig. 1, the reaction rate is faster at the beginning since almost 75% of the conversion of the two substrates into the desired product is achieved in the first 24 hours. After one day, the concentrations of the two reactants become extremely low, so that the reaction rate dramatically slows down. This translated in complete conversion being obtained only after about 200 hours.

Complete conversions are achievable in shorter times using a twofold excess of the amino component (98% in 144 hours), as reported in Table 1. Nevertheless, employment of equimolar concentrations of the reactants is recommended, since this enables the recovery and isolation of the products simply by withdrawing the organic phase and evaporating the toluene, thus avoiding any purification step. As a consequence, high isolated yields are achievable (>90%).

In conclusion, the present work describes the thermodynamically controlled enzymatic synthesis of aspartame precursors performed in a hydrophobic organic solvent, leading to complete conversions and to high isolated yields even when using equimolar concentrations of the reactants.

This opens new perspectives for the large-scale production of biologically active peptides using peptidases in low-water media. Moreover, the results presented in this paper confirm that Celite R-640® is an effective tool for productive biocatalysis in low-water media, and is potentially applicable to large-scale processes.

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