High isolated yields in thermodynamically controlled peptide synthesis in toluene catalysed by thermolysin adsorbed on Celite R-640

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An innovative immobilisation method that allows peptide synthesis to be performed even at equimolar concentrations, by controlling water activity, is reported.

Enzymes have proved to be an attractive alternative to chemical methods in peptide synthesis since several proteases effect peptide bond formation under mild conditions, with minimum side-chain protection, and avoiding racemization. In spite of these benefits, enzymatic methods are not routinely employed, partly because they suffer from unfavourable thermodynamics in water.

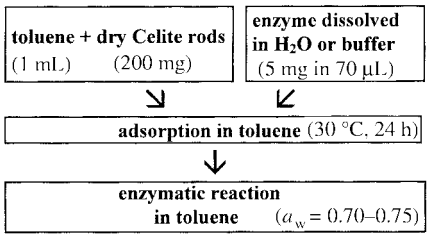
There are two distinct mechanisms for protease-catalysed peptide synthesis: thermodynamically and kinetically controlled synthesis. In principle, replacing water with organic solvents as a reaction medium should be beneficial for both types of reaction. However, water is essential for proteases such as thermolysin and α-chymotrypsin, and various studies confirmed that these enzymes display no activity when insufficiently hydrated.[1] Unfortunately, water/solvent mixtures are not optimal media for peptidases since high percentages of co-solvents reduce the activity of the enzyme dramatically.

Owing to these two opposite roles of water in enzymatic peptide synthesis, various approaches have been developed in order to search for compromises. These include kinetically controlled syntheses in solvents containing low percentages of water [2] or methods to reverse the equilibrium towards synthesis, such as using an excess of the amino component and extracting/precipitating the product.[3]

Kitaguchi and Klibanov[1a] have given an alternative solution to the problem by demonstrating that water can be replaced partially by water mimics such as formamide, maintaining the activity of the enzyme in the organic solvent while minimising hydrolytic reactions. Finally, promising results have been obtained by Halling and coworkers by controlling the hydration of thermolysin in kinetically controlled peptide synthesis in hexane[4] and, more recently, by developing solid-to-solid peptide synthesis in the presence of minimum amounts of water.[1b]

Aiming to overcome the above drawbacks to a larger application of enzymes in peptide synthesis, the present work describes a novel approach for the non-covalent immobilisation of thermolysin in toluene and its application to thermodynamically controlled peptide synthesis at controlled water activity (a_w). The method exploits the ability of Celite R-640 rods (Fluka) to adsorb large amounts of water (> 90% of its weight) and to maintain the a_w constant in a reaction system within wide defined ranges of water concentrations.[5,6] Thermolysin was adsorbed on dry Celite R-640 according to an innovative and practical technique illustrated in Scheme 1. The enzymatic aqueous solution was added to the hydrophobic solvent containing the dry support. Owing to the presence of the hydrophobic solvent, a uniform coating of the aqueous phase formed around the Celite rods and the enzymatic solution was adsorbed on the Celite within 24 h.

The immobilised catalyst suspended in toluene gave, after equilibration, a_w values between 0.70 and 0.75. Enzymatic reactions can be carried out simply by adding the reactants to the toluene used for the adsorption and storage of the enzymes. The use of the hydrophobic organic solvent makes the leakage of the enzyme negligible so that the biocatalyst can be recycled. The hydrophobic solvent leads to further remarkable advantages in terms of stability, especially if compared to the detrimental effects of the water miscible solvents largely reported.[7] For instance, the hydrolases immobilised on Celite R-640 in toluene were stored in the same solvent at a controlled degree of hydration without any appreciable decrease of activity for at least 4 weeks. The same immobilisation technique can also be employed for the immobilisation of α-chymotrypsin and penicillin G amidase (PGA), which were shown to be effective in catalysing amino acid esterification and protection of amino groups, respectively. Table 1 reports some examples of syntheses catalysed by the three immobilised enzymes in toluene at controlled a_w. The water adsorbed by the porous support provides the enzymes with the hydration necessary to display their catalytic activity.[2–4] It should be noted that no reaction was observed when the three native enzymes were employed in dry toluene or acetonitrile in accordance with our observation that PGA is also active in organic solvents only when sufficiently hydrated (a_w > 0.4).[5]



Scheme 1

Table 1 Enzymatic synthesis performed in toluene at a_w between 0.70 and 0.75 at 30 °C

Enzyme[a]	CO_2H donor[b]	Nucleophile[b]	Final conversion[c] (%)	*t*/h
Thermolysin/Celite	Z-L-Phe–CO_2H	L-Phe–OEt	98	48
Thermolysin/Celite	Z-L-Phe–CO_2H	L-Tyr–OEt	97	144
Thermolysin[d]/Celite	Z-L-Phe–CO_2H	L-Leu–NH_2[d]	95	96
PGA[e]/Celite	$PhCH_2CO_2Me$	L-Tyr–OEt	98	24
α-Chymotrypsin[e]/Celite	Z-L-Phe–CO_2H	MeOH	97	60

[a] Enzyme dissolved in buffer and adsorbed on 200 mg of Celite rods according to Scheme 1. [b] Equimolar concentrations of reactants (80 μmol) were employed in all reactions. An excess of MeOH (250 μmol) was used only in the esterification of Z-L-Phe-CO_2H. [c] Conversions calculated by means of RP-HPLC using an internal standard. Isolated yields of Z-L-Phe-L-Phe–OEt, Z-L-Phe-L-Tyr–OEt and Z-L-Phe-L-Leu–NH_2 were 93, 90 and 76%, respectively. Reactions catalysed by α-chymotrypsin and thermolysin were monitored also by titrating the Z-L-Phe–CO_2H with 0.1 M NaOH solution. [d] The enzyme was dissolved in 35 μL of buffer, adsorbed on the Celite rods and equilibrated for 24 h. Afterwards, a buffer solution (35 μL) containing L-Leu–NH_2 was added to the thermolysin/Celite system and equilibrated for 3 h. [e] The enzyme was dissolved in water before adsorption.

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The use of Celite R-640 as a support for thermolysin and α-chymotrypsin enables peptide bond synthesis and esterification to be performed using free carboxylic acids since the water produced during the reaction is adsorbed by the Celite rods. Therefore a_w was maintained sufficiently low and hydrolytic reactions were prevented so that nearly quantitative conversions were achieved even when employing equimolar concentrations of non-activated, and thus less expensive, substrates. It must be noted that when native thermolysin was employed in water–acetonitrile (13% v/v)† in the synthesis of Z-L-Phe-L-Tyr–OEt, a maximum of 22% of conversion was obtained in 6 h but during the reaction course a_w exceeded 0.90 so that hydrolytic reactions prevailed, causing a very low final conversion (11%).

Despite the fact that the poor solubility of peptides has often been viewed as a restriction for enzymatic reactions in organic solvents, results in Table 1 indicate that thermodynamically controlled peptide enzymatic synthesis in hydrophobic solvents can be carried out employing substrates having very low solubility and which are present in the reaction medium mainly as a suspension. This observation is in accord with previous studies concerning kinetically controlled peptide synthesis[4] and the solid-to-solid synthesis.[1b,c]

The dipeptide Z-L-Phe-L-Leu–NH_2 was synthesised following an alternative strategy since the poorly soluble L-Leu–NH_2 was previously dissolved in an aqueous buffer and then adsorbed on the Celite rods where thermolysin had been already immobilised. However, this second method led to lower isolated yields (76%) probably owing to some adsorption of the product on the Celite rods.

The time required to achieve complete conversion is mainly affected by the fact that equimolar concentrations of the reactants were used in all peptide synthesis, so that the reaction rate slows down dramatically at the end of the reaction when the concentrations of the two reactants become extremely low. Complete conversions are achievable in shorter times using a two fold excess of the amino component (> 98% yield of Z-L-Phe-L-Tyr–OEt in 48 h). Nevertheless, employing equimolar concentrations of reactants is advisable since this enables the recovery and isolation of the products very simply and, most importantly, avoiding any purification step.‡ As a consequence, remarkably high isolated yields (93 and 90%) are achievable. This is a factor of major importance in peptide synthesis, especially when various subsequent synthetic steps are required.

Reactions reported in Table 1 catalysed by α-chymotrypsin and PGA are also of practical use in peptide synthesis since they are potentially useful for the preparation and protection of activated amino acids suitable for kinetically controlled peptide synthesis. It is noteworthy that α-chymotrypsin catalysed the complete esterification of Z-L-Phe, despite it being previously reported that the enzyme accepts preferentially the *N*-acetyl amino acids,[1d] which, however, are deprotected with difficulty.

The novelty of our method lies not only in the synthetic results obtained but also in the immobilisation technique developed. The use of Celite R-640 allows control of the water activity, rather than water concentration, during the whole process, thus controlling effectively both the enzyme activity and the reaction equilibrium. No devices, such as hydrated salts,[8] equilibration with the atmosphere at known relative humidity,[9] or more complex methods[10] for adjusting the a_w of the system are required; also there is no need to remove the water produced during the process.[11] Furthermore, the immobilisation method fulfils the fundamental requirements for a larger application to biotransformations since the procedure is simple and inexpensive, it provides very high reproducibility in terms of enzyme and water content so leading to reproducible activity and quantitative adsorption yields. Detrimental effects caused by the removal of water under vacuum or with polar solvents[12–14] are avoided. Moreover, microbial contaminations are prevented, the support material is stable under the reaction conditions, and possesses no swelling capacity.

In conclusion, the present work describes the first example, to the best of our knowledge, of thermodynamically controlled enzymatic peptide synthesis performed in organic solvent leading to complete conversions and very high isolated yields even when equimolar concentrations of the reactants are employed.

Since it has been already demonstrated that peptidases catalyse the formation of the bond between oligopeptide fragments in organic solvents and that PGA is able to remove phenylacetic groups selectively from amino acids, we are currently investigating the application of this technique to a totally enzymatic approach for peptide synthesis competing with chemical routes.

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Notes and references

† 13% v/v of H_2O enables operation at $a_w \approx 0.73$[12d] in MeCN.

‡ The three synthesised dipeptides precipitated upon formation owing to their low solubility in toluene. The toluene was removed and the solid residue was washed with MeCN. The organic solutions were combined, filtered and taken to dryness obtaining products having > 98% purity by HPLC. Products of reactions catalysed by PGA and α-chymotrypsin are soluble in toluene and were isolated by removing the organic phase and evaporating the solvent.

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