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Protease-Catalyzed Peptide Synthesis on Solid Support

Rein V. Ulijn,[†] Beatriz Baragaña,[†] Peter J. Halling,[‡] and Sabine L. Flitsch*,[†]

Department of Chemistry, The University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JJ, Scotland, and Department of Chemistry, The University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, Scotland

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The direct enzymatic synthesis of peptides from amino acids is widely used as a useful alternative to chemical synthesis. However, good yields of such enzyme-catalyzed reactions require altered reaction conditions to overcome the preference for hydrolysis in aqueous medium. For example, organic (co-) solvents[1] and highly concentrated substrate suspensions[2] have been used to shift the equilibrium toward synthesis.[3] Both of these methods have their drawbacks and limit the scope of the biotransformation. In this report, we demonstrate that the equilibrium can also be shifted toward synthesis *in aqueous medium* by immobilizing the amine on solid support (Scheme 1). Thus, we report the first examples of protease-catalyzed high-yielding peptide synthesis on solid support in bulk aqueous buffer.

It is well known that the synthesis of amides from free amines and carboxylic acids is thermodynamically highly unfavorable in dilute aqueous media. Various "low-water" reaction media have been developed to shift this reaction equilibrium toward synthesis. The most intensively studied approach consists of (partly) replacing water with organic solvents.[1] This approach offers several advantages, and many applications of protease-catalyzed peptide synthesis in such reaction media have been reported. A drawback is that enzyme activity is often significantly lower and substrate solubility can be limiting. Recently, an additional type of "low-water" reaction medium has been used which gave good results in peptide synthesis when the reaction product precipitated from aqueous substrate suspensions.[2] A drawback of these "solid-to-solid" reactions is that product precipitation (and high yields) is not thermodynamically favored for all reactions.[2c]

There is a considerable current interest in using enzymes on solid supported substrates in screening of combinatorial libraries,[4] chemoenzymatic synthesis,[5] and enzyme cleavable linkers.[6] Meldal and co-workers have developed special supports, such as PEGA (poly(ethylene glycol)-acrylamide), that have been shown to be useful for these applications because they are accessible to small enzymes.[7] For example, proteases have been employed to hydrolyze solid supported peptides for identification of their substrate specificity and in screening for inhibitors.[7] The feasibility of employing proteases for solid-phase peptide synthesis has, however, not been reported before.

There are several reasons why a shift in equilibrium toward synthesis can be expected when the amine substrate is linked to a solid support. One widely recognized advantage of solid-phase synthesis is that large excesses of substrates can be used to help drive reactions to completion. A second contribution is expected from suppressed ionization of the solid supported amine due to the overall positive charge of the resin.[8] A final effect may result from the improved solvation of hydrophobic acyl donors in PEGA resin when compared to aqueous environment.[9] This increase will result in a local higher concentration of acyl donor near the site of catalysis.[10] For these reasons, we believed solid-phase peptide synthesis via the direct reversal of the hydrolytic reaction would be feasible, and we put this hypothesis to the test.

Scheme 1. Amide Synthesis/Hydrolysis on Solid Supported Substrate

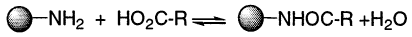
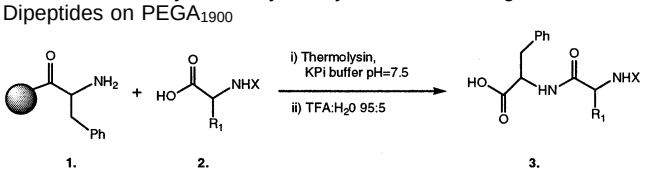


Table 1. Thermolysin-Catalyzed Synthesis of a Range of Dipeptides on $PEGA_{1900}$



acyl donor[a]	product[b]	X	R_1	conversion (%)[b]
2a	**3a**	Fmoc	H	99
2b	**3b**	Fmoc	$CH_2-CH-(CH_3)_2$	99
2c	**3c**	Fmoc	$CH_2-C_6H_5$	99
2d	**3d**	Cbz	$CH_2-C_6H_5$	72
2e	**3e**	Fmoc	D-$CH_2-C_6H_5$	0
2c,**2e**	**3c**	Fmoc	D/L-$CH_2-C_6H_5$	99[c]
2f	**3f**	Fmoc	$CH_2-CH_2-CONH_2$	84
2g	**3g**	Fmoc	CH_2-OH	10
2h	**3h**	Fmoc	$CH_2-(imidazole)H^+$	77
2i	**3i**	Fmoc	CH_2-COO^-	70
2j	**3j**	Fmoc	$CH_2-(CH_2)_2-CH_3$	99

[a] Unless specified, L amino acids were used. [b] Yields determined by HPLC. [c] Only the L,L-dipeptide was detected by HPLC and LC-MS. A mixture of both diastereoisomers prepared chemically was used as the standard.

Thermolysin was chosen as a suitable biocatalyst, because it is known to be robust, and it is a commonly used enzyme in thermodynamically controlled peptide synthesis.[11] Because of its broad specificity for the carboxylic acid substrate, thermolysin can be employed to catalyze a variety of peptide synthesis reactions with acyl donors with different polarities and functionalities.

Thus, Fmoc-phenylalanine was coupled to $PEGA_{1900}$ (Polymer Laboratories, UK) via the Wang linker, and the amino group was then deprotected with piperidine. Resin bound phenylalanine (**1**) was then treated with excess carboxylic acids (**2a**−**2j**) in the presence of thermolysin. Reaction products (**3a**−**3j**) were released from the resin by acid cleavage and quantified by HPLC. Table 1 summarizes the results. With hydrophobic acyl donors (entries **2a**−**d**, **2j**), very good yields were observed. These results demonstrated that the shift in equilibrium for amide formation on solid phase can be sufficient to yield complete conversion to the amide.

* To whom correspondence should be addressed. E-mail: S.Flitsch@ed.ac.uk.
[†] The University of Edinburgh. Fax: +44 (0)131 650 4737. Tel: +44 (0)131 650 4737.
[‡] The University of Strathclyde. Fax: +44 (0)141 552 4822. Tel: +44 (0)141 548 2683.

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The solubility of the Fmoc amino acids is very low in aqueous buffer (generally <1 mM), but this did not limit the yields observed. Indeed, better yields were observed when the acyl donors were more hydrophobic. For example, with Cbz-Phe (**2d**), the observed yield (72%) for the phenylalanine dipeptide was still good but considerably lower. This observation may be explained in terms of a lower hydrophobic contribution to the shift in equilibrium. Indeed, for polar substrates (**2f**−**i**), the yields of **3f**−**i** were also lower.

In conventional peptide synthesis, an excess of 5−10-fold of amino acid is generally required for complete coupling. It is interesting to note that excess of acyl donor required in our studies compares favorably (4-fold excess needed for complete conversion of **2j** to **3j**) with chemical methods. However, we expect that the excess required will depend on the aqueous solubility of the Fmoc-amino acid and will be higher for more polar amino acids. We are currently working on a model that aims to quantify relative contributions from suppressed ionization, substrate solvation, and these substrate excess effects. With increased understanding, the yields of some of these reactions may be improved further in the future.

Entries **3f**−**i** illustrate a major advantage of using enzymes for peptide synthesis instead of conventional Fmoc chemistry in that side chains of these amino acids do not need to be protected, even when the side chains are significantly basic (**3h**) or acidic (**3i**).

A further advantage of using enzymes in synthetic applications is their high enantioselectivity. Entry **3c** shows that it was possible to synthesize the L,L-diastereoisomer with high selectivity when a DL-mixture of Fmoc-Phe (**2c**,**2e**) was presented as the acyl donor.

It is well established that certain proteases can be used to catalyze peptide formation involving nonnatural amino acids. This is shown here by using Fmoc-norleucine (**2j**) successfully as an acyl donor to generate **3j** in excellent yield.

The reactions in Table 1 were all conducted for 14 h to ensure equilibrium was reached. However, in subsequent studies we have found that shorter reaction times (e.g., of 2 h for synthesis of **3c**) are sufficient for complete conversion. Preliminary kinetic studies suggest an initial rate of reaction at about 0.1 μmol min^{-1} mg^{-1}, which is around 1 order of magnitude lower than that of thermolysin-catalyzed synthesis in aqueous solution. The difference can be explained in terms of lower rates of diffusion when the substrates are linked to solid supports.

In summary, we have reported the first examples of high-yielding protease-catalyzed peptide synthesis on solid support. It is particularly interesting to note that the reactions can be conducted in bulk aqueous medium, with no need for organic cosolvent or activated carboxylic acid. We are currently studying the scope of this reaction for the synthesis of larger peptides.

Solid-phase substrates were linked to PEGA$_{1900}$ (Polymer Laboratories) beads via a Wang-type linker (hydroxymethylphenoxyacetamide). PEGA$_{1900}$-Phe was prepared using standard Fmoc chemistry in DMF.

For the enzymatic reactions, 10 mg of PEGA$_{1900}$ (100 mg of wet weight stored in MeOH, loading 0.2 mmol/g dry polymer) was washed with 2 × 5 mL of 0.1 M potassium phosphate buffer of pH 7.5. Five milligrams of thermolysin (protease type X from Sigma) was added to a suspension of the washed PEGA$_{1900}$ resin, 0.2 mmol protected amino acid, and 2 mL of 0.1 M potassium phosphate buffer of pH 7.5. Reactions were briefly mixed and subsequently incubated overnight at room temperature on a blood rotator. The next day, the resin was washed extensively using 5 mL volumes in the following sequence: 5× DMF, 5 × 50:50 (v/v) DMF:MeOH, 5× MeOH, 5 × 50:50 (v/v) acetonitrile/water, 5× MeOH. The products were cleaved from the resin with 2 mL of TFA:water 95:5 during 2 h. Resin was then washed with 10 mL of a mixture of 50:50 acetonitrile in water with 0.1% TFA, solvent was evaporated off, and the residue was redissolved in 1 mL of a 50:50 mixture of acetonitrile and water. The samples were analyzed by HPLC (Waters 2690 LC system equipped with a Waters 468 UV detector) and by LCMS (Waters 2790 LC system coupled with a Micromass Platform II mass spectrometer using Electrospray ionization mode).

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(8) It is well known that the favored hydrolysis of amides in dilute aqueous solutions is largely due to the favorable ionization of amino acids. When the equilibrium constant for peptide synthesis is expressed in terms of the un-ionized forms of the reactants only, the value was shown to be $10^{3.6}$ M^{-1}, largely towards synthesis. See: Ulijn, R. V.; Moore, B. D.; Janssen, A. E. M.; Halling, P. J. *J. Chem. Soc., Perkin Trans. 2* **2002**, *5*, 1024−1028. Suppression of ionization leads to a shift of equilibrium toward synthesis, as observed when organic cosolvents are used. For solid supported amine substrates, ionization of amino groups is significantly suppressed by the proximity of positive charges of neighboring amines. An equilibrium shift towards synthesis is expected. An alternative picture is that amide synthesis destroys positive amine charges on the resin and reduces the unfavorable repulsion between them.

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