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Enzymatic optical resolution *via* acylation–hydrolysis on a solid support

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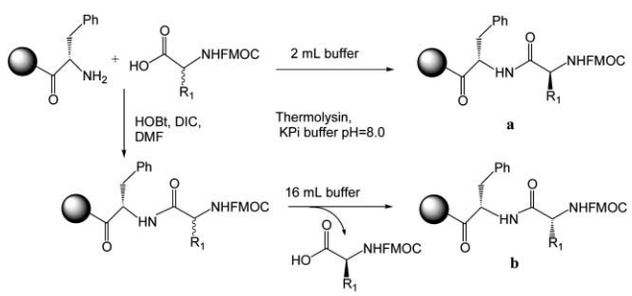
By taking advantage of the reversibility of the thermolysin-catalysed amide synthesis–hydrolysis reaction on a solid support, both L,L and L,D diastereoisomers of dipeptides and L-amino acids are accessible in good yields starting from enantiomeric mixtures of amino acids.

Enzymes, in particular lipases and acylases, are now established as useful catalysts for the generation of optically pure compounds.[1] In aqueous media, hydrolysis is thermodynamically favoured and enzymes can be used for optical resolutions of enantio- and diastereoisomers. The reverse reactions, stereoselective acylations, are also possible but they require modified reaction conditions to overcome unfavourable thermodynamics. Media that are now commonly used for these reactions include organic solvents or ionic liquids.[2] Stereoselective transacylation reactions have also been described, either in aqueous media or in the presence of co-solvents. These reactions require activated compounds such as esters or amides instead of free acids as substrates.[3]

There are several examples of the enzyme-catalysed hydrolysis of amide or ester bonds of solid-supported substrates, such as for example in solid phase screening for protease substrates.[4] We have recently demonstrated that under certain conditions the reverse reaction (enzyme-catalysed acylations) on a solid support are also feasible in bulk aqueous media.[5] Hence, by tuning the reaction conditions both acylation and hydrolysis reactions are feasible on a solid phase. Scheme 1



Scheme 1 Thermolysin-catalysed synthesis (1a) or hydrolysis (1b) to access both diastereomeric dipeptides.

shows how enzyme enantiospecificity can be exploited in such systems to access both diastereoisomeric dipeptides *via* acylation (Scheme 1a) or hydrolysis (Scheme 1b) from racemic mixtures of amino acids.

In our previous report, the protease thermolysin was shown to catalyse peptide synthesis on a $PEGA_{1900}$ (poly-(acrylamide)-ethylene glycol) support, often in good yields.[5] It was also shown that when a racemic mixture of Fmoc-Phe was used as the reagent, only the L,L diastereoisomer was formed with very high stereospecificity (Scheme 1a). Such stereospecific enzyme-catalysed reactions on a solid support had not been exploited before and it was felt that these preliminary results warranted further investigations.

Table 1 Thermolysin-catalysed enantioselective synthesis of a range of dipeptides on $PEGA_{1900}$.† Unless otherwise indicated 10 equivalents of soluble Fmoc amino acid per immobilised Phe were used in 2 mL 0.1 M Kpi buffer at pH 8. The fourth column that is headed by 'D/L' gives the optical rotation of the soluble Fmoc amino acid

Entry	R_1	Time/h	D/L	%	de (%)
1a	CH_2-C_6H_5	228	D/L	99	99
1b[a]	CH_2-C_6H_5	16	D/L	99	99
1c	CH_2-C_6H_5	48	L	99	—
1d	CH_2-C_6H_5	64	D	< 0.1	—
2a	$(CH_2)_3$-CH_3	64	D/L	99	99
2b	$(CH_2)_3$-CH_3	48	L	99	—
2c	$(CH_2)_3$-CH_3	64	D	< 0.1	—

[a] Instead of 10 equivalents of soluble Fmoc amino acid per solid Phe, 100 equivalents were used in this reaction. This did not affect the yield but did appear to increase the rate of the reaction.

As a first step, the amount of excess of Fmoc amino acid required for the reaction was investigated. In our previous studies a 100-fold excess of free Fmoc-Phe over solid-supported phenylalanine substrate was used. Such a high excess would clearly prohibit applications. However, due to the low aqueous solubilities of Fmoc-protected amino acids (generally less than mM) a significant proportion of the excess added remained undissolved throughout the course of these reactions, suggesting that complete conversions could be obtained with a much smaller excess of acyl donor present.

Indeed, for the synthesis of Fmoc-L-Nle-L-Phe and Fmoc-L-Phe-L-Phe a 10-fold excess was sufficient to reach complete conversions to the L,L peptide from soluble L-amino acids (Table 1: 1c, 2b) or racemic mixtures (1a, 1b, 2a). In addition, no L,D diastereoisomer could be detected when a racemate or only the D-amino acid was supplied (1d, 2c).

These numbers compare well with 5–10 fold excess required for chemical peptide synthesis. It should be noted that the present enzymatic methodology is compatible with chemical synthesis methods (where also Fmoc-protected amino acids are used). Our present approach might therefore be interesting in cases where the L-amino acids are not readily available or expensive, since resolution of enantiomers and coupling can be achieved in one step.

Next, the kinetic resolution of a dipeptide on a solid phase was investigated by making use of the reverse (hydrolysis) reaction. This would be complementary to the previous technique of stereoselective dipeptide synthesis since it woud generate the L,D-diastereoisomer (Scheme 1b) on a solid support and would release enantiopure Fmoc-L-amino acid into solution.

Initial hydrolysis experiments on solid-supported Fmoc-L-Phe-L-Phe were conducted under standard conditions (using 20 mg resin with a loading of 0.1 mmol g^{-1}‡ in 2 ml aqueous buffer, 1 μmol of solid-supported substrate per ml of buffer, Table 2, entry 3a). Even after 4 days, the yields of hydrolysis were disappointing (52%). It seemed unlikely that this low yield

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Table 2 Thermolysin catalyzed enantioselective hydrolysis of a range of dipeptides on $PEGA_{1900}$.§ The fourth column that is headed by 'D/L' gives the optical rotation of the Fmoc amino acid initially present on the solid phase

Entry	R_1	*V*/ml	D/L	μmol ml^{-1} [a]	%[b]	ee (%)
3a	CH_2-C_6H_5	2	L	1	52	—
3b	CH_2-C_6H_5	6	L	0.33	62	—
3c	CH_2-C_6H_5	6	L	0.16	92[c]	—
3d	CH_2-C_6H_5	16	L	0.13	87	—
3e	CH_2-C_6H_5	16	D/L	0.13	48	99
3f	CH_2-C_6H_5	16	D	0.13	< 0.1	—
4a	$(CH_2)_3$-CH_3	16	L	0.13	70	—
4b	$(CH_2)_3$-CH_3	16	D/L	0.13	43	99
4c	$(CH_2)_3$-CH_3	16	D	0.13	< 0.1	—

[a] Amount of solid-supported substrate per buffer volume. [b] All reactions were left at RT for 72 hours. [c] In this reaction half the amount of $PEGA_{1900}$ was used.

was due to low enzyme activity, since the same enzyme had been able to catalyse the reaction in the synthesis direction in good yield.

An obvious explanation based on our previous studies was the unfavourable position of the equilibrium. The effect of reaction volume on yield of hydrolysis was therefore further investigated. Indeed, hydrolysis yields could be increased by using larger reaction volumes (corresponding to 0.33 (entry 3b), 0.16 (3c) and 0.13 (3d) μmol of solid-supported substrate per ml of buffer) which yielded up to 87–92% of hydrolysis product. Further increase in volume did not increase the yields further and hence the incomplete conversion is probably not fully limited by equilibrium.

Once the reaction conditions for optimal hydrolysis were established the kinetic resolution of L/D,L peptides was investigated. Thus, after chemical synthesis of a mixture of dipeptide diastereomers Fmoc-D/L-Phe-L-Phe and Fmoc-D/L-Nle-L-Phe on $PEGA_{1900}$ (Scheme 1b) thermolysin was added in 16 mL phosphate buffer at pH 8.0. After 72 hour incubation at room temperature the supernatant was analysed for the Fmoc-Phe or Fmoc-Nle by chiral HPLC.¶ In each case, only the L enantiomer could be detected and conversions were nearly complete (3e, 4b). The remaining compounds on $PEGA_{1900}$ resin were then cleaved off the solid support using TFA and analysed by LCMS. In each case only the D,L diastereoisomer could be detected, close to the theoretical maximum of 50%, confirming that all L,L peptide was completely cleaved (3e, 4b). In addition, the L,D diastereoisomer was not cleaved at all (3e, 3f, 4b, 4c).

An advantage of carrying out acylation and hydrolysis reactions on immobilised substrates is that the separation of desired and undesired reaction products is straightforward by filtration. This allows for the easy recovery of both solid phase peptides and soluble amino acids.

There have only been very few reports on solid phase applications of enzyme-catalysed kinetic resolutions in the literature.[6] More general use has so far been restricted because of poor yields, explained by poor swelling of the resin and resulting problems of substrate accessibility to the enzyme. In our present work we have overcome such problems by using $PEGA_{1900}$, a resin which has much better swelling properties in water. However, we have shown that hydrolysis yields are also determined by equilibrium effects that are different for supported compared to soluble substrates.

Thus, by taking advantage of the reversibility of the thermolysin-catalysed amide synthesis–hydrolysis reaction on a solid support, both L,L and L,D diastereoisomers of dipeptides and L-amino acids are accessible in good yields starting from enantiomeric mixtures of amino acids.

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Notes and references

† Solid phase substrates were linked to $PEGA_{1900}$ (Polymer Laboratories) beads as described previously.[5]

‡ The loading quoted by the supplier was 0.2 mmol g^{-1}. In our hands a maximum of 0.1 mmol g^{-1} peptide could be coupled.

§ In hydrolysis reactions, Fmoc-protected amino acid was coupled with $PEGA_{1900}$-Phe chemically by using carbodiimide activation methods. 3 mg thermolysin and 6 ml 0.1 M potassium phosphate buffer of pH 8.0 were added to the washed $PEGA_{1900}$-Phe-Phe/Nle-Fmoc resin. The reaction mixture was rotated for 72 hours on a blood rotator. Afterwards the resin was washed with 10 mL of a mixture of 50 : 50 acetonitrile in water, solvent was evaporated off and the residue redissolved in 1 mL 50 : 50 mixture of acetonitrile and water. Solid phase amino acids were analysed as described before.[5]

¶ Chiral HPLC was performed on a Waters 2690 LC system equipped with a Waters 468 UV detector equipped with an OD-H Chiralcel column.

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