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Model Studies on the Utility of Nucleophiles Bound to Insoluble Supports for Enzymatic Peptide Synthesis

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Thermolysin, α-chymotrypsin, and papain were used as biocatalysts for the coupling of several carboxyl components to silica-supported leucine amide as a nucleophile. A spacer length of 21 covalent bonds corresponding to 7 amino acid residues was required for successful coupling. This approach offers the possibility of using proteases for racemization-free segment condensations on insoluble polymeric supports.

(Keywords: α-Chymotrypsin; Enzymatic peptide synthesis; Papain; Solid-phase peptide synthesis; Thermolysin)

Modellstudien zur Anwendbarkeit von an unlösliche Träger gebundenen Nucleophilen für die enzymatische Peptidsynthese

Thermolysin, α-Chymotrypsin und Papain wurden als Biokatalysatoren für die Kupplung verschiedener Carboxylkomponenten an kieselgel-gebundenes Leucinamid als Nucleophil eingesetzt. Für erfolgreiche Kupplungen war eine Spacerlänge von 21 kovalenten Bindungen entsprechend 7 Aminosäureresten erforderlich. Diese Synthesevariante eröffnet die Möglichkeit, racemisierungsfreie Segmentkondensationen an unlöslichen polymeren Trägern mit Hilfe von Proteasen durchzuführen.

Introduction

During the last decade there has been a renewed interest in the application of proteolytic enzymes to peptide bond formation (reviews 1-3). The main adavantages of enzyme catalysis are i) the high optical purity of the products, ii) the minimal side chain protection required, and iii) the mild reaction conditions and minimization of side reactions which are often observed and which complicate chemical couplings. In most cases the equilibrium is shifted towards synthesis by precipitating

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the products[4] by means of a large excess of the amino component[5] and by addition of high concentrations of water-miscible cosolvents[6] (thermodynamic approach). Kinetically controlled peptide bond formation with the products remaining in solution is possible utilizing esterolytic enzymes in conjunction with esters as carboxyl components, and an excess[7,8] or, more economically, a nearly equimolar amount of nucleophile[9,10].

Hitherto, all the protease-catalyzed peptide bond formation experiments employing both soluble and immobilized[10,11] enzymes have been carried out with amino components soluble in the reaction medium. In this communication we wish to report on the results of model studies using silica-supported leucine amide as a nucleophile for protease-mediated peptide bond formation.

Abbreviations: IUPAC/IUB rules for peptides were followed, see Eur. J. Biochem. **27**, 201 (1972); *Aca-* = 6-aminohexanoyl, *Boc* = *tert.*-butyloxycarbonyl, *Z* = benzyloxycarbonyl, —O*Su* = *N*-hydroxysuccinimide ester. —O*Me* = methyl ester, *AP* = 3-aminopropyl, *AP*Si = 3-aminopropyl silica, *DCC* = *N*,*N'*-dicyclohexylcarbodiimide, HO*Bt* = 1-hydroxybenzotriazole.

Materials and Methods

α-Chymotrypsin (EC 3.4.21.1, 45 U/mg), papain (EC 3.4.22.2, 12 U/mg), and thermolysin (EC 3.4.24.4, 40 U/mg) were commercially available from Worthington (Freehold, U.S.A.), Merck (Darmstadt, FRG), and Boehringer (Mannheim, FRG), and were used as received. Silica gel 60 (70–230 mesh) used as support material was a product of Merck. *Z*-Leu-Phe-O*Me*[11] and *Z*-Phe-Leu-O*Me* (yield 80%, m.p. 108–109 °C from ethyl acetate/petroleum ether, $[\alpha]_D^{22} = -24.7°$, $c = 2$ in *Me*OH; Ref. [12] gives: m.p. 111–113 °C, $[\alpha]_D^{25} = -24.4°$, $c = 0.5$ in *Me*OH) were synthesized by the mixed anhydride method as described in Ref. [11]. *Boc*-Leu-O*Su*[13] was prepared from *Boc*-Leu-OH and *N*-hydroxysuccinimide by means of *DCC*. All other reagents and solvents used were either reagent grade quality or were purified and dried prior to use. Potentiometric titration was accomplished by Radiometer (Copenhagen, Denmark) electrodes P 4011 and K 601.

Boc-Leu-Aca-OH: was obtained from *Boc*-Leu-O*Su* and H-*Aca*-O$^-$ in 1,4-dioxan/water (4:1, *v*/*v*), overall yield 47%, m.p. 97–100 °C, $[\alpha]_D^{22} = -20.8°$, $c = 1$ in *Me*OH. Anal. calcd. (found): C 59.28 (60.02), H 9.53 (9.36), N 8.13 (8.01%).

Silanization: of silica gel 60 with 5% (*v*/*v*) 3-aminopropyltriethoxy silane in toluene (5 ml/g silica) according to Ref. [14] yielded a material containing 0.764 mmol NH_2-groups per g support, as determined by potentiometric titration[15].

H-*Leu-AP*Si: was synthesized by coupling of *Boc*-Leu-OH (2.31 g, 10 mmol) with 2.5 g (1.9 mmol NH_2-groups) *AP*Si by means of *DCC* (2.06 g, 10 mmol) in 40 ml CH_2Cl_2 for 6 h. After three couplings the loading amounted to 69% (0.526 mmol *Boc*-Leu-groups/g support). Residual amino groups were blocked

with Ac_2O/pyridine (50 ml, 1:10, *v/v*) in the presence of catalytic amounts (50-70 mg) of 4-*N*,*N*-dimethylaminopyridine[16]. The *Boc*-protecting groups were released with 1.2 *M*-HCl in acetic acid (3 equivalents, 0.5 h with occasional shaking). Yield 0.443 mmol H-Leu-groups/g (84% of the *Boc*-groups removed). The use of additives such as HO*Bt* for the coupling is not recommended because of the strong adsorption of these compounds at the silica and the serious difficulty to remove them.

H-*Leu-Aca-AP*Si: 2.5 g (1.9 mmol NH_2-groups) *AP*Si, 2.3 g (6.7 mmol) *Boc*-Leu-*Aca*-OH, and 1.4 g (6.7 mmol) *DCC* in 40 ml CH_2Cl_2 gave, after stirring for 38 h, a loading of 69% (0.526 mmol/g). Residual amino groups were blocked as above, and removal of the *Boc*-protection resulted in 0.412 mmol H-Leu-*Aca*-groups/g (78%).

H-(*Leu-Aca*)$_2$-*AP*Si: was obtained by acylation of 1.5 g (0.62 mmol H-Leu-*Aca*-groups) H-Leu-*Aca-AP*Si with 2.1 g (6.2 mmol) *Boc*-Leu-*Aca*-OH and 1.28 g (6.2 mmol) *DCC* in 30 ml CH_2Cl_2 for 38 h. The loading was 98% [0.402 mmol *Boc*-(Leu-*Aca*)$_2$-groups/g]. Removal of the *Boc*-groups proceeded quantitatively yielding 0.402 mmol H-(Leu-*Aca*)$_2$-groups/g silica.

Protease-Mediated Coupling Reactions

For all experiments 250 mg samples corresponding to 0.111 mmol H-Leu-, 0.103 mmol H-Leu-*Aca*-, and 0.10 mmol H-(Leu-*Aca*)$_2$-groups bound to silica were used.

Thermolysin: 2.1 mg (0.056 μmol) enzyme, 67.1 mg (0.224 mmol) *Z*-Phe-OH, 2 ml tris-maleate-buffer (0.2 *M*, *pH* 7.0), and the nucleophile were stirred for 22 h. After careful washing (buffer, 1 *M*-NaCl, *Me*OH) the content of free amino groups was determined.

α-*Cymotrypsin*: 10 mg (0.4 μmol) enzyme, 95.5 mg (0.222 mmol) *Z*-Leu-Phe-O*Me*, 1 ml CCl_4 plus 2 ml *Michaelis*-buffer (0.2 *M*, *pH* 8.5), and the silica-supported amino component were stirred for 6 h and treated as above.

Papain: 60 mg (2.57 μmol) enzyme, 95.5 mg (0.222 mmol) *Z*-Phe-Leu-O*Me*, 0.5 ml *Me*OH plus 2.4 ml *McIlvaine*-buffer (0.2 *M*, *pH* 5.5), 0.1 ml mercaptoethanol for activation of the enzyme, and the nucleophile were stirred for 4 h and treated as described for thermolysin.

With α-chymotrypsin and papain, t.l.c. analysis showed almost exclusively hydrolysis of the ester substrates after the coupling experiments.

Results and Discussion

For the model studies silica gel was selected as support material for the amino component because of its inertness. It is relatively stable to treatment with common organic solvents and in aqueous solution and it does not swell. The amide linkage to the support was preferred over the ester linkage commonly used for solid-phase peptide synthesis in order to avoid any hydrolytic cleavage of the bound nucleophile under the reaction conditions used for the enzyme-mediated coupling experiments.

Table 1 summarizes the results of the coupling experiments with thermolysin, α-chymotrypsin, and papain as catalysts and different carboxyl components to H-(Leu-*Aca*)$_2$-*AP*Si as amino component. The choice of the reaction conditions and of the carboxyl components followed the most successful experiments employing soluble leucine amide as a nucleophile[11,17,18] but in general the enzyme concentration and the reaction time was increased.

Table 1. *Protease-mediated peptide bond formation with H-(Leu-Aca)$_2$-APSi as amino component*

	Protease (carboxyl component)		
	Thermolysin (*Z*-Phe-OH)	α-Chymotrypsin (*Z*-Leu-Phe-O*Me*)	Papain (*Z*-Phe-Leu-O*Me*)
Yield (%, mmol/g)	60, 0.242[a]	20, 0.081	0, 0

[a] After the second coupling.

Similar experiments with H-Leu- and H-Leu-*Aca*-*AP*Si as amino components were completely unsuccessful and within the error of determination (± 3%) no consumption of amino groups could be measured after the coupling experiments.

The main reason for the failure of couplings to leucine bound to the silica by shorter spacers is most probably the inaccessibility of the nucleophile to the enzymes due to unfavourable steric interactions between the enzyme protein and the silica surface. The close proximity required for binding of the terminal leucine to the active S_1' site of the enzymes which is necessary for peptide bond formation seems to be impossible for steric reasons, hence the substrates were cleaved only hydrolytically and no peptide bond formation could be observed. In accordance with this interpretation, chemically synthesized *Z*-Phe-Leu-*Aca*-*AP*Si was not cleaved by the three enzymes. In addition, supporting this view, amino acid and peptide derivatives bound to polyethyleneglycol, a soluble and conformationally flexible support, are freely accessible to enzymes in aqueous solution[19,20].

In the experiments with H-(Leu-*Aca*)$_2$-*AP*Si as amino component the spacer, -*Aca*-Leu-*Aca*-*AP*-, consists of 21 covalent bonds corresponding to 7 amino acid residues: this length was required for successful protease-mediated coupling experiments (cf. Tab. 1). A reasonable loading of 46% in a single thermolysin-catalyzed coupling

step was obtained with a ratio of carboxyl component to bound leucine of 2:1. The maximum yield was 60% after repeated coupling, a third coupling gave no yield improvement. The chymotrypsin-catalyzed coupling of *Z*-Leu-Phe-O*Me*, employing the aqueous-organic two-phase approach[11,21,22], gave a loading of only 20% *Z*-Leu-Phe-(Leu-*Aca*)$_2$-groups, whereas papain failed to catalyze the coupling with *Z*-Phe-Leu-O*Me* at all. Both enzymes have esterase activity, and acylation by ester substrates is very fast. In the competition between water and the nucleophile for the acylenzymes water is strongly favoured due to the restricted mobility of the silica-bound amino component. Consequently, the acylenzymes undergo mainly or exclusively cleavage by hydrolysis. Under the reaction conditions applied, repeated formation of acyl-enzymes from the dipeptide acids *Z*-Leu-Phe-OH and *Z*-Phe-Leu-OH formed by hydrolysis will be very slow, i.e. the reaction stops, especially within the short reaction times used. In the case of thermolysin, an enzyme without any esterolytic properties, hydrolysis of the enzyme intermediate generates the original substrate, *Z*-Phe-OH, which then may react again with the enzyme.

Silica is a porous material with cavities of different sizes. During the the chemical modification of the silica, groups are also anchored in smaller pores and cavities being inaccessible to the large high molecular weight enzymes despite of the long spacer used. In our opinion, this is a reasonable explanation for the fact, that a loading of 60% was the maximum with thermolysin as a catalyst.

Conclusions

It has been shown that silica-supported leucine amide may be a useful amino component for protease-mediated peptide bond formation provided that the spacer is sufficiently long. With short spacers the coupling reactions are prevented by steric interactions between the enzyme protein and the silica surface; the bound nucleophile cannot bind to the S_1' position of the active center of the enzyme necessary for peptide bond formation. Although not studied, such contacts might be of importance in preventing the enzymic hydrolysis of the ester linkage commonly used for reversible attachment of the growing peptide in syntheses on insoluble polymeric supports. The use of carboxyl-unprotected substrates is recommended in connection with long reaction times due to "recycling" of the substrate. Ester substrates, which react much faster with soluble amino components, were mainly cleaved by hydrolysis and gave lower coupling yields with the immobilized nucleophile.

Based on these results, segment condensations on insoluble polymeric supports could be achieved successfully, in principle, by protease-catalysis without the risk of racemization. A serious difficulty seems to be the large size of the biocatalyst, which only reacts with groups bound to the surface or located in large cavities of the support, whereas groups anchored in smaller pores and cavities are inaccessible to the enzyme (depending on the length of the spacer).

Since amino acids and peptides bound to the soluble polyethyleneglycol support are freely accessible to enzymes[19,20] the use of proteases for segment condensations in liquid-phase peptide synthesis seems to be more convenient employing the kinetic approach. In addition, there will be no unfavourable restriction in the mobility of the amino component bound to the soluble support, and immobilized proteases could be used as catalysts. Experiments along this line are in progress.

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