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Peptide Synthesis by Means of Immobilized Enzymes

I. Immobilized α-Chymotrypsin

Andreas Könnecke*, Ralf Bullerjahn,
and **Hans-Dieter Jakubke**

Sektion Biowissenschaften, Bereich Biochemie, Karl-Marx-Universität,
DDR-701 Leipzig, German Democratic Republic

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α-Chymotrypsin covalently bound to silica, enzacryl AA, and enzacryl AH catalyzes peptide bond formation between N-protected dipeptide methyl esters and H-Leu-NH_2 with results similar to those with the free enzyme. The influence of water-miscible and water-immiscible cosolvents, of the supports, and of the structure of the substrates is shown to be of importance for the ease of the chymotrypsin-medicated coupling reactions. The best yields were obtained using biphasic aqueous-organic solvent mixtures, silica-bound chymotrypsin, and substrates with leucine in the P_2-position. The yields of the syntheses are discussed in terms of the reactivity of substrates with similar structure in enzymatic hydrolyses. All the immobilized chymotrypsin preparations could be re-utilized successfully for further couplings.

(Keywords: Biphasic aqueous-organic solvent mixtures; α-Chymotrypsin; Immobilized enzymes; Peptide synthesis)

Peptidsynthesen mit immobilisierten Enzymen. I. Immobilisiertes α-Chymotrypsin

Kovalent an Kieselgel, Enzacryl AA und Enzacryl AH gebundenes α-Chymotrypsin katalysiert die Peptidbindungsknüpfung zwischen N-geschützten Dipeptid-Methylestern und H-Leu-NH_2 mit ähnlichen Ergebnissen wie das freie Enzym. Es wird gezeigt, daß mit Wasser mischbare und nichtmischbare Lösungsmittel, die Trägermaterialien sowie die Struktur der Substrate den Verlauf der Chymotrypsin-katalysierten Kupplungsreaktionen beeinflussen. Die besten Ausbeuten wurden in wäßrig-organischen Zweiphasensystemen mit Kieselgel-gebundenem Chymotrypsin und mit Substraten mit Leucin in P_2-Position erhalten. Die Syntheseausbeuten werden in bezug auf die Reaktivität von Substraten ähnlicher Struktur bei der enzymatischen Hydrolyse diskutiert. Alle immobilisierten Chymotrypsin-Präparationen konnten mit Erfolg für weitere Kupplungsreaktionen wiedergenutzt werden.

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Abbreviations: IUPAC/IUB rules for peptides are followed, see Eur. J. Biochem. **27**, 201 (1972); Glt = 4-carboxybutyryl (glutaryl), -Nan = 4-nitroanilide. All amino acids except glycine are of L-configuration.

Introduction

Following the classical work of *Bergmann* et al. concerning the reversal of protease-catalyzed peptide bond hydrolysis[1], a renewed interest in application of proteolytic enzymes to peptide synthesis as an alternative to existing chemical methods arose during the last years[2,3]. One of the main advantages of the coupling reactions catalyzed by proteases is the high optical purity of the products due to the stereospecificity of the enzymes, and a variety of well-known proteolytic enzymes has been used previously[2,3].

The basic idea of all bond forming processes catalyzed by proteolytic enzymes is, that it should be possible, in principle, to replace the water acting in hydrolysis by other reagents such as amines or alcohols. In the case of chymotrypsin, a serine protease, definite acyl enzyme intermediates are formed during catalysis by acylation of Ser-195[4]. Deacylation of these intermediates by water is equivalent to hydrolysis of the substrates, while alcoholysis or aminolysis affords new synthetic products. For several acyl-chymotrypsins formed from N-blocked amino acids it has been shown that the rate of hydrolysis is much lower than the rate of aminolysis by C-blocked amino acid derivatives[5,6], implying the feasibility of peptide bond formation. This has been verified experimentally utilizing soluble chymotrypsin as a catalyst for peptide synthesis[7–9]. Furthermore, if water is formed during acylation of the enzyme (with N-protected amino acids or peptides) the equilibrium is shifted towards synthesis by either addition of high concentrations of water-miscible organic cosolvents due to perturbations of the pK-values of the reactants[10] or by using aqueous-organic biphase systems where the low water content decreases the rate of hydrolysis of the formed product[11].

Hitherto, immobilized chymotrypsin was used successfully for esterification of N-protected amino acids[11–14], but initial attempts to build up peptide bonds by means of immobilized chymotrypsin in 76% (v/v) ethanol failed[15].

In a recent preliminary communication we reported on the usefulness of silica-bound α-chymotrypsin for peptide bond formation[9] and here we report on the extension of these results. The aim of the present investigation was to study the influence of (i) the cosolvents (either water-miscible or immiscible), (ii) the supports, and (iii) the structure of

the substrates on the ease of the coupling reactions catalyzed by immobilized α-chymotrypsin. Furthermore, results of re-utilization experiments of the enzyme preparations will be pesented.

Materials and Methods

General Remarks

Bovine α-chymotrypsin (EC 3.4.21.1) (3 times crystallized and salt free) was purchased from Boehringer (Mannheim, FRG) or from Spofa (Prague, Czechoslovakia). Amino acids were obtained from Reanal (Budapest, Hungary) and the enzacryls from Koch and Light (UK). All other chemicals and reagents used were either reagent grade quality or were purified and dried prior to use. Thin layer chromatography (t.l.c.) was performed on silica-coated alumina foils (Kavalier, Czechoslovakia) using at least two different solvent systems for

Table 1. *Yields and properties of the dipeptide methyl ester substrates*

Substrate	Yield[a] (%)	m.p.[a] (°C)	Optical rotation $[\alpha]_D^{22}$ (degrees)	Literature values
Z-Gly-Phe-O*Me*	68	Oil	+ 6.8 ($c = 4.2$, *Me*OH)	
Z-Ala-Phe-O*Me*	80	100-102	−12.2 ($c = 2$, *Me*OH)	m.p. 103-104[16]; 96-97[17]
Z-Val-Phe-O*Me*	62	141	−26.9 ($c = 2$, *Me*OH)	
Z-Leu-Phe-O*Me*	65	83-84	−27.0 ($c = 1$, *Me*OH), $[\alpha]_{546}^{22}$ −31.0	m.p. 81, $[\alpha]_{546}^{20}$ −26.8 ($c = 1$, *Me*OH)[18]
Z-Pro-Phe-O*Me*	71	68-71	−37.2 ($c = 2$, *Me*OH)	
Z-Phe-Phe-O*Me*	86[b]	146-148	−16.7 ($c = 0.4$, *Et*OH)	m.p. 148-150[19]; 132-133, −16.6 ($c = 0.4$, *Et*OH)[20]
Z-Leu-Tyr-O*Me*	72[c]	88-92	−15.5 ($c = 2.5$, *Me*OH)	
Boc-Leu-Phe-O*Me*	76	88-89	−30.3 ($c = 1$, *Et*OH)	
Ac-Leu-Phe-O*Me*	78	115-116	−38.6 ($c = 1$, *Et*OH)	

[a] From *Et*O*Ac*/petroleum ether.
[b] From *Et*OH/petroleum ether.
[c] Noncrystalline material, recrystallization was impossible due to gel formation in several solvents.

checking homogeneity (see Table 2). Melting points were determined with an *Boetius* apparatus and are corrected. Optical rotations (Polamat A, 1 dm cells) are accurate to at least ± 0.5°. Nuclear magnetic resonance (n.m.r.) spectra were run using a Tesla BS 487 C instrument operating at 80 MHz with *TFA* as solvent for the analysis of the mixtures (see Table 4). Elemental analyses for all compounds mentioned were within acceptable limits.

Substrates

The dipeptide methyl esters were synthesized by the mixed anhydride method using isobutyl chloroformate and N-ethyl morpholine as reagents and tetrahydrofuran or ethyl acetate as solvents. In order to obtain optically pure

products, the coupling reactions were performed at —30 °C for 1 h and then for 1 h at room temperature (time for anhydride formation between 2 and 3 min). As a representative example, the synthesis of *Z*-Pro-Phe-O*Me* is described in detail.

To a stirred solution of 4.99 g (20 mmol) *Z*-Pro-OH in 80 ml of dry ethyl acetate 2.52 ml N-ethyl morpholine were added at —30°. After about 10 min 2.60 ml isobutyl chloroformate were introduced and after further 2 min a precooled solution of H-Phe-O*Me*, made by dissolving 4.32 g (20 mmol) H-Phe-O*Me* · HCl in a small amount of water, adding 2.52 ml N-ethyl morpholine and then about 30 ml ethyl acetate, was added. The stirring was continued for 1 h at —30° and then for 1 h at room temperature, whereafter the solution was washed carefully with water, 1 *N*-HCl, water, saturated $NaHCO_3$ solution, and again with water followed by drying over anhydrous Na_2SO_4. Removal of the ethyl acetate left a viscous oil which crystallized after storage for 8 d at 4°. Recrystallization of this material from ethyl acetate/petroleum ether yielded 5.83 g (71%) *Z*-Pro-Phe-O*Me* as white crystals, m.p. 68–71°, $[\alpha]_D^{22}$ —37.2° ($c = 2$, *Me*OH).

Ac-Leu-Phe-O*Me* was obtained by acetylation of H-Leu-Phe-O*Me* · HCl. made from *Boc*-Leu-Phe-O*Me* with a twofold excess 1.2 *N*-HCl in acetic acid, with pyridine/acetic acid anhydride (1 : 1, *v/v*) (40-fold excess) for 1 h. Yields and physical properties of the substrates are summarized in Table 1. H-Leu-NH_2 and H-Leu-NH_2 · HCl utilized as nucleophiles were made by ammonolysis of H-Leu-O*Me* or H-Leu-O*Me* · HCl in methanol.

Immobilization

α-Chymotrypsin was covalently bound to γ-succinamidopropyl silica by the N-hydroxysuccinimido ester method[21]. For the experiments listed in Table 3 the support material was macroporous silica (pore diameter 20 nm) and the content of bound chymotrypsin was 20 mg/g dry silica according to amino acid analysis. Experiments of Table 4 were run using silica gel 60 (Merck) as support (30 mg/g). Standard procedures were followed for the immobilization on enzacryl AA and AH[21, 22] (21.0 and 42.5 mg/g determined by the difference method).

The hydrolytic activity of all preparations was assayed using Glt-Leu-Phe-Nan as substrate[23]. The retained activity of the immobilized enzymes was found to range between 75 and 90% of the activity of the native enzyme.

Enzymatic Coupling Reactions

The reaction mixtures each contained 0.2 mmol ester substrate, 0.2 mmol H-Leu-NH_2 as nucleophile, immobilized chymotrypsin, the appropriate cosolvent, and 0.2 *M*-carbonate buffer (*pH* 10), in the ratio indicated in Tables 3 and 4. The choice of the organic cosolvent was mainly governed by the solubility of the substrates and the amount used often represents the minimum to dissolve it. The mixture was then vigorously stirred at room temperature (ca. 25°) by means of an overhead stirrer (magnetic stirring destroys the silica) for the time indicated. The N-protected tripeptide amide formed precipitated and after filtration it was removed from the immobilized enzyme by short treatment with several portions of either methanol or precooled *DMF*. The filtrate obtained was evaporated to dryness in vacuo, treated with water and set aside for at

least 6 h at 4° to ensure complete crystallization of the product; the remaining immobilized enzyme was carefully washed with doubly distilled water and stored for re-utilization in doubly distilled water at 4°. The product was then collected on a weighed sintered glass filter, successively washed with 1 *N*-HCl, water, and saturated $NaHCO_3$ solution, and dried in vacuo to constant weight. After the homogeneity had been checked by t.l.c. the yield was determined by weight; estimated error from several parallel runs $\leq \pm 3\%$. In several cases it was neccessary to wash the crude product with some small portions of CCl_4 in

Table 2. *Properties of the products obtained by the coupling reactions catalyzed by immobilized α-chymotrypsin*

Product	m.p.[a] (°C)	$[\alpha]_D^{22}$ solvent *Me*OH	R_F-values[b] A	B	C
Z-Gly-Phe-Leu-NH_2	209–211	—20.1 ($c = 1$)	0.31	0.13	0.16
Z-Ala-Phe-Leu-NH_2	218–219[c]	—50.5 ($c = 1$)	0.35	0.11	0.19
Z-Val-Phe-Leu-NH_2	274–276	—41.3 ($c = 0.3$)	0.49	0.21	0.20
Z-Leu-Phe-Leu-NH_2	226–231	—53.1 ($c = 1$)	0.61	0.43	0.47
Z-Pro-Phe-Leu-NH_2	186–187	—77.7 ($c = 0.72$)	0.43	0.16	0.25
Z-Leu-Tyr-Leu-NH_2	235–238	—45.3 ($c = 0.55$)	0.34	0.14	0.25
Boc-Leu-Phe-Leu-NH_2	205–208	—49.0 ($c = 0.45$)[d]	0.71	0.49	0.59
Ac-Leu-Phe-Leu-NH_2	275–278	—54.8 ($c = 1$)[e]	0.42	0.21	0.33

[a] After one crystallization from *Me*OH/water.
[b] Solvent systems: A, $CHCl_3$/acetone/*Me*OH (7:2:1); B, $CHCl_3$/*n*-propanol (9:1); C, $CHCl_3$/*Me*OH (9:1).
[c] Ref. 7 gives m.p. 228–230°.
[d] Solvent *Et*OH.
[e] Solvent *Ac*OH.

order to remove unreacted starting ester, some product was also lost during this procedure.

In order to facilitate comparison, standard reaction conditions were used for the experiments of Table 4: 0.2 mmol ester substrate, 0.4 mmol H-Leu-NH_2·HCl, time 1.5 h, total volume 2.5 ml, amount of immobilized enzyme corresponding to 3 mg (0.12 μmol) chymotrypsin, and removal of the product from the immobilized enzyme by *DMF*. The actual *pH* in the buffer phase was 8.5–8.7.

The crude products, if not contaminated with starting ester, were obtained essentially pure differing in their properties insignificantly from the once crystallized products (Table 2). No attempts were made to optimize the yields.

Results and Discussion

Influence of the Cosolvents

Three examples demonstrating the influence of cosolvents on the yields of the coupling reactions catalyzed by immobilized chymotrypsin are presented in Table 3. In all cases the best yields were obtained

using biphasic aqueous-organic solvent mixtures, i.e. water-immiscible cosolvents, as already reported for soluble proteases[9]. In the presence of methanol or *DMF* as cosolvents the yields were significantly lower, whereas polyethyleneglycol (*PEG*) gave intermediate yields.

A large amount of data is already available concerning the influence of organic cosolvents on various aspects of enzyme action, especially for chymotrypsin[24, 25]. Although no comparison has been made before, it seems that the catalytic process is much more strongly influenced by the direct contact of the enzyme with water-miscible cosolvents of high dielectric constant than by the apolar water-immiscible cosolvents interacting preferentially by hydrophobic mechanisms with the

Table 3. *Influence of cosolvents on the coupling reactions catalyzed by immobilized α-chymotrypsin*

Substrate	Cosolvent (%, *v/v*)	Total volume (ml)	Time (h)	Amount of bound enzyme (mg)	Yield (%)
Ac-Leu-Phe-O*Me*	PEG (55)[a]	2.2	1.75	3	49
Ac-Leu-Phe-O*Me*	CH_2Cl_2 (60)	2.5	1.5	2.5	75
Ac-Leu-Phe-O*Me*	CH_2Cl_2 (60)	2.5	1.5	2	64
Boc-Leu-Phe-O*Me*	*Me*OH (36)	2.2	2	3	8[b]
Boc-Leu-Phe-O*Me*	PEG (55)[a]	2.2	2.5	4	24[b]
Boc-Leu-Phe-O*Me*	CCl_4 (50)	2	3	4	32[b]
Z-Ala-Phe-O*Me*	DMF (35)	2	2	3	31
Z-Ala-Phe-O*Me*	PEG (57)[a]	2.3	2	3	50
Z-Ala-Phe-O*Me*	CCl_4 (56)	2.7	2	3	70

[a] PEG refers to polyethyleneglycol 600.
[b] After washing with CCl_4 to remove unreacted starting ester substrate.

enzyme. Furthermore, a variety of water-miscible solvents act as competitive inhibitors[25], and although no detailed information is available for water-immiscible solvents their inhibitory properties are thought to be much lower.

Besides the solvent-enzyme interactions discussed above the solubility of the product will be considerably lower in the buffer phase of the biphasic aqueous-organic solvent mixtures than in reaction mixtures containing water-miscible cosolvents. This means, that the rate of hydrolysis of the once formed product, especially at stages of the reaction where most of the starting ester is consumed and the product competes the with remaining ester substrate for the enzyme, will be lowered in biphasic systems.

Nearly all applications of enzymes in organic synthesis require the addition of organic cosolvents to augment the solubility of the substrates, and water-miscible solvents such as *DMF*, *DMSO*, acetonitrile. acetone, methanol, ethanol etc. are most commonly used for this purpose[24, 25]. Although a very broad discussion concerning advantages of biphasic aqueous-organic solvent systems for enzyme-catalyzed reactions has appeared, only one experimental example was reported in this paper[11]. Furthermore, the partition coefficients of the substrates between the two immiscible phases[11] are only one aspect bearing on the outcome of the overall reaction in such complex systems. Nevertheless, the results presented in this paper as well as results from other fields of enzymatic transformations[26] are encouraging and clearly demonstrate the advantages of the uses of aqueous-organic solvent mixtures for synthetic application of enzymes. Finally it should be noted that our experimental approach using immobilized enzymes as catalyst in biphasic solvent systems is closely related to the so-called triphase catalysis[27], which uses insoluble catalysts to accelerate aqueous-organic phase reactions.

Influence of the Supports and of the Substrate Structure

Since it is well known that the catalytic activity of immobilized enzymes is strongly influenced by the kind of the support material and by the mode of binding of the enzyme to the support, we investigated three immobilized chymotrypsin preparations in which the enzyme was covalently bound to the support. The binding was achieved by either acylation of lysyl-ε-amino groups (silica and enzacryl AH) or by azo coupling with tyrosine residues (enzacryl AA) of the enzyme protein.

The results listed in Table 4 show that all three immobilized chymotrypsins are useful for peptide bond formation. For a given substrate the yield depends on the kind of catalyst, i.e. the support material. In nearly all cases the silica-bound enzyme provided the best yields suggesting that this is the most useful catalyst for such synthetic purposes. But at the present stage of investigation no general conclusion can be drawn with a view to designing a "good" catalyst, because unspecific interactions between the substrates and the supports also have to be taken into consideration. Such interaction will depend mainly on the structure of the substrate and is most probably responsible for the observation that in some cases better yields were obtained with the enzacryl-bound enzyme.

Despite these unspecific interactions mentioned above, the substrate structure chiefly influences the binding to the enzyme. Consequently, the structure of the substrates will be of general importance

for the ease of enzyme-mediated coupling reactions. Using standard reaction conditions as for the experiments listed in Table 4, only two variables greatly influence the rate of the coupling reaction, i.e. the yield, for a given catalyst, (i) the structure of the substrate, and (ii) the kind and the amount of the water-immiscible cosolvent.

In the *Z-Xxx*-Phe-O*Me* series studied the variation of the amino acid residue *Xxx* in the P_2-position (nomenclature according to ref. 28)

Table 4. *Influence of the support materials and of the substrate structure on the coupling reactions catalyzed by immobilized α-chymotrypsin*[a]

Substrate (cosolvent, %, *v/v*)	Silica	Support material Enzacryl AH Yield (%)[b]	Enzacryl AA
Z-Gly-Phe-O*Me* (CCl_4, 20)	59 (57)	61 (64)	51 (50)
Z-Ala-Phe-O*Me* (CCl_4, 40)	59 (60)	—	49 (54)
Z-Val-Phe-O*Me* ($CHCl_3$, 20)	79 (79)[c] 52[d]	81 (77)[c] 18[d]	69 (68)[c] 36[d]
Z-Pro-Phe-O*Me* (CCl_4, 20)	54 (57)	—	60 (59)
Z-Leu-Phe-O*Me* (CCl_4, 12)	88 (88)	75 (75)	61 (63)
Z-Leu-Tyr-O*Me* (CCl_4, 20)	61 (57)	—	47 (44)

[a] For standard reaction conditions see Materials and Methods.
[b] The yields of two independent runs are given.
[c] Crude yield of products contaminated by starting ester substrate.
[d] Average product yield in the crude product according to n.m.r. analysis (integration of the sum of the benzylic protons of the *Z*-groups vs. the methyl group protons of the unreacted ester substrate) and the yields of product after hydrolyzing the starting ester in the mixture with 1 *N*-NaOH in *Me*OH and determining the actual yield of product by weight. Both methods agreed within ± 5%.

slightly influences the yields. The best yields were obtained with the substrate containing Leu in P_2-position and the silica-bound enzyme (cf. Table 4).

Some studies on the secondary specificity of chymotrypsin revealed that the steric strain exerted by the isobutyryl side chain of Leu with Ile-99 of the enzyme in P_2-S_2 contacts enhances the reactivity of synthetic peptide substrates[29], chromogenic substrates[23], and inhibitors[30] towards the enzyme, mainly due to increasing k_{cat} values. For the

amide bond hydrolysis k_{cat} stands for the rate-limiting formation of the acyl-enzyme from the *Michaelis* complex, and if the validity of the principle of microscopic reversibility is supposed, the reserve reaction, i.e. the aminolysis of the acyl-enzyme in peptide synthesis, also should be accelerated providing higher yields (under standard conditions). Furthermore, the S_2 subsite of the enzyme was shown to accept a variety of apolar side chains in P_2-position without significant changes in the reactivity of the substrates[29, 31–33] agreeing with the comparable yields obtained for the other substrates with Gly, Ala, or Pro in the P_2-position.

In contrast, the S_3 subsite of chymotrypsin is much more discriminating with respect to side chains in P_3-position[33–35]. For example, the reactivity of α-aminoacyl aldehyde inhibitors with *Ac*- or *Tfa*-groups in P_3 was found to be about 100-fold higher compared with the corresponding *Boc*-derivative[33]. Hence, the low reactivity of our *Boc*-Leu-Phe-O*Me* substrate most probably accounts for the poor yield in comparison to the *Ac*- or *Z*-dipeptide methyl esters of the same sequence (see Tables 3 and 4). Since the kinetic analyses revealed a three- to fourfold higher reactivity of substrates with Tyr in P_1-position compared with Phe in P_1[31, 36], the lower yields of the couplings with *Z*-Leu-Tyr-O*Me* relative to *Z*-Leu-Phe-O*Me* cannot be explained satisfactorily at present.

As shown by the kinetic deacylation experiments with several acyl-chymotrypsins[6] as well as by the kinetics of peptide hydrolyses[36, 37], the enzyme possesses a peculiar P′-specificity (leaving group specifity in hydrolytic processes). The S′-subsites are hydrophobic accepting hydrophobic P′-residues and the highest deacylation rates were measured with amino acid amides having hydrophobic or aromatic side chains as nucleophiles[6, 37], free amino acids were found to be less reactive[6]. Moreover, for a given nucleophile the deacylation rate also depends on the acyl moiety bound in the acyl-chymotrypsin[6].

Again, there is experimental material available[7] showing that the yields of couplings catalyzed by soluble chymotrypsin roughly correlate with the deacylation rates of the acyl-chymotrypsins; H-Leu-NH_2 and H-Val-NH_2 provided the best yields whereas free amino acids failed to couple.

In conclusion, the detailed knowledge of the specificity of a proteolytic enzyme may be useful tool in predicting the success of a planned coupling reaction.

Interestingly, attempted coupling of *Z*-Phe-Phe-O*Me* with H-Leu-NH_2 resulted in the formation of *Z*-Phe-Leu-NH_2 as main product due to the unexpected cleavage of the Phe—Phe bond of the substrate. As

with *Z*-Val-Phe-O*Me* the reaction with *Z*-Phe-Phe-O*Me* was incomplete after the standard reaction time of 1.5 h and not all of the starting ester was consumed, which may be due to the 20% $CHCl_3$ used as cosolvent, dictated by the low solubility of both substrates in CCl_4. On extending the reaction time to 5 h, the reaction was still incomplete and a careful t.l.c. analysis with reference compounds made chemically (mixed anhydride method for *Z*-Phe-Leu-NH_2 and *Z*-Phe-Phe-Leu-NH_2) indicated *Z*-Phe-Leu-NH_2 to be the main reaction product and that only minor amounts of the expected *Z*-Phe-Phe-Leu-NH_2 were formed. These results might be regarded as an illustration of the complexity of effects influencing the coupling reactions catalyzed by proteolytic enzymes. On the other hand, *Ac*-Pro-Ala-Phe-Phe-NH_2 is also hydrolyzed at the Phe—Phe bond by chymotrypsin in addition to hydrolysis of the terminal amide bond[29].

Re-utilization Experiments

The intrinsic possibility of reusing immobilized enzymes in industrial processes, both continuously or discontinuously, represents the main aspect in research in this area besides the general scientific interest regarding the properties of immobilized enzymes compared

Table 5. *Results of re-utilization experiments*[a]

Substrate	Support material	Cosolvent	Solvent for removal of the product	Yield (%) 1st	2nd re-utilization	3rd
Ac-Leu-Phe-O*Me*	Silica	*PEG*	*Me*OH	49	52	48
Ac-Leu-Phe-O*Me*	Silica	CH_2Cl_2	*Me*OH	75	71	72
Ac-Leu-Phe-O*Me*	Silica	CH_2Cl_2	*DMF*	75	65	—
Ac-Leu-Phe-O*Me*	Silica	CH_2Cl_2	*Me*OH	64	62	—
Z-Leu-Phe-O*Me*	Silica	CCl_4	*DMF*	88	76	71
Z-Leu-Phe-O*Me*	Enzacryl AA	CCl_4	*DMF*	63	57	44
Z-Gly-Phe-O*Me*	Enzacryl AH	CCl_4	*DMF*	64	60	57

[a] After the first use the immobilized enzyme was stored in doubly distilled water for about 4–6 weeks at 4°, whereafter the 2nd re-utilization experiment was performed under the same conditions as described for the 1st use, and this cycle was followed exactly for the 3rd re-utilization.

with the native enzymes[38, 39]. Although our reactions are not suitable for continuous processing because the products precipitate from the reaction mixture, we have studied the possibility of re-utilizing the immobilized enzymes for further coupling reactions. The results collect-

ed in Table 5 clearly demonstrate that all the immobilized chymotrypsins could be re-utilized successfully for at least three times by discontinuous batching. Notably, the solvent used for removal of the product from the immobilized enzyme plays an important role in these experiments. Whereas methanol is obviously advantageous for this purpose because the yields remained nearly constant up to the third re-utilization of the enzyme, with *DMF* the yields decreased significantly. Clearly, the *DMF* treatment inactivates the immobilized chymotrypsin much more than contact with methanol. It may be that the use of e.g. *DMSO* for which it has been shown that the enzyme remains active in high concentrations of this solvent[40] also would be advantageous for removal of the product from the immobilized enzyme.

Conclusions

It has been shown that immobilized chymotrypsin is a useful catalyst for peptide bond formation. Under nearly identical conditions the reaction times are somewhat longer than with the free enzyme, but the amount of enzyme required for complete reaction seems to be often lower using the immobilized enzyme[41]. Advantages of the use of immobilized enzymes in peptide synthesis are (i) the enzyme can be re-utilized for further coupling reactions, (ii) the reaction products are nearly pure, not contaminated with either proteolytic activities or denatured protein often observed utilizing soluble proteases[41], and (iii) as with soluble proteases the coupling reactions catalyzed by immobilized chymotrypsin also provide products of highest obtainable optical purity. For example, the N,N-dicyclohexylcarbodiimide/1-hydroxybenzotriazole mediated coupling of *Ac*- or *Boc*-Leu-Phe-OH with H-Leu-NH_2 yielded products of lower optical purity, $[\alpha]_D^{22}$ —44.3° ($c = 0.5$, *Et*OH) for the *Boc*-, and $[\alpha]_D^{22}$ —50.6° ($c = 0.25$, *Ac*OH) for the *Ac*-tripeptide amide (cf. Table 2).

As discussed previously in ref. 20, there are some serious disadvantages of enzyme-mediated couplings, especially the difficulty of predicting whether a certain coupling can be achieved successfully. As shown above this difficulty can be overcome, at least partially, if the specificity of the enzyme is known in detail, because there is obviously a strict relationship between the rate of hydrolysis of enzyme substrates and the rate of re-synthesis. Furthermore, the reactor volumes can be kept small using aqueous-organic biphase systems, this approach generally also providing the best yields.

In some cases it has been shown that the equilibrium between synthesis and hydrolysis is shifted kinetically controlled towards synthesis, for example in the protease-catalyzed semi-syntheses of

human insulin[42], and that it is not always neccessary to remove the product by precipitation from the equilibrium. Certainly, the catalysis of such kind of reactions represents the main perspective for the utilization of immobilized proteases in peptide synthesis.

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