Proteases in Organic Synthesis†

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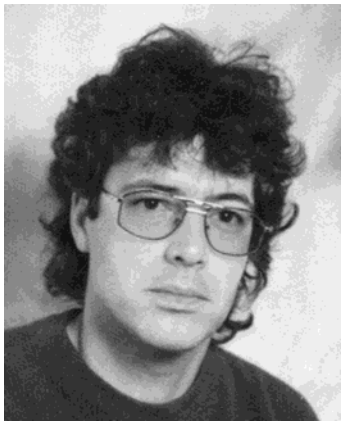

Frank Bordusa was born in 1969 in Markranstädt (Saxony), Germany. He studied Biochemistry at the University of Leipzig where he graduated with a diploma in 1995. He completed his Ph.D. degree with Professor H.-D. Jakubke on enzyme kinetics and specificities of the bacterial cysteine protease clostripain in 1998. He then moved to Professor G. Fischer's Max-Planck Research Unit for Enzymology of Protein Folding in Halle/Saale where he is currently working on his Habilitation. His research centers on bioorganic and synthetic peptide chemistry, especially the development of novel enzymatic and chemoenzymatic routes to the synthesis of peptides, peptide mimetics and proteins by substrate, enzyme, and medium engineering.

I. Introduction

The synthetic utility of proteases has been recognized long before their chemical nature was known. In 1783, Spallazani postulated from his experiments that gastric juice bears an intrinsic catalytic activity mediating the digestion of meat.[1] It took another 128 years until Hofmeister[2] first recognized the basic architecture of proteins consisting of amide bond-linked amino acid monomers—an elementary finding that opened the door to a more rational investigation of enzymes. The following decades were shaped by enthusiastic and fundamental investigations on the function and structure of enzymes whereby proteases often played a prominent role. As a result, proteases are among the best characterized enzymes, and much of the current knowledge of structure and function of proteins has been derived from those investigations.[3] Historically, proteases have generally been associated with protein digestion. In contrast, it might be surprising that already at the end of the 19th Century the first experimental hints to a reverse action of those enzymes were found, a phenomenon that was originally termed "plastein-reaction"[4,5] and that was predicted several years earlier by van't Hoff.[6] However, from this first point until now, where proteases are considered normal chemical bench reagents in organic synthesis, it was a long way, which is still far away from being terminated.

This account is mainly focused on the last five years of this impressive development of using proteases in organic synthesis. A simple search in the Ovid database, for example, provides a rough estimation of the scientific activities in this research field. About 10% of all papers dealing with proteases are more or less connected with a synthetic use of these enzymes. Furthermore, compared to 1996 to 2000 an increase of the total number of publications by about 45% is evident. For synthetic use, only lipases and esterases give a higher number of database entries. Accounting proteases, lipases, and esterases together makes hydrolases predominant among the reported

† Dedicated to Prof. P. Welzel on the occasion of his 65th birthday.

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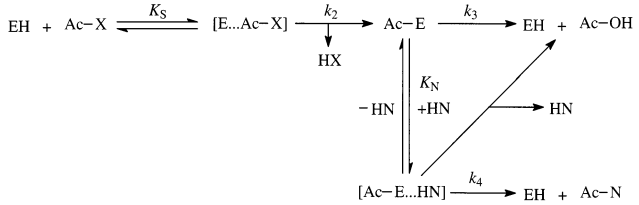
Scheme 1. Equilibrium-Controlled Proteases Synthesis

enzymes for organic synthesis. This large number of papers alone makes it quite impossible to write a review covering all efforts and achievements in this field, so this review is necessarily selective. The about 600 papers included are those which, in the opinion of the author, have general applicability or particular significance. Furthermore, no particular attention will be paid to subjects that have been recently reviewed in detail. In such instances, only the most important trends are briefly summarized. Discussion of basic aspects of protease action and their inherent synthetic activity is also reduced to a minimum to avoid redundance to the long series of papers already published in journals or books.[7]

II. General Aspects of Protease-Catalyzed Reactions

Proteases, or following the recommendation of the NC−IUB *peptidases*, belong to the enzyme class of hydrolases and are one of the only few enzymes that are active at the backbone of polypeptides. Currently, several hundred of these enzymes are known, and, in a general sense, they all catalyze the same reaction: hydrolysis of peptide bonds. One could ask why such a relatively nonspectacular activity might be interesting for organic chemists. Analysis of the characteristics of this activity provides a plausible explanation. Proteases are highly stereo- and regiospecific catalysts that usually act under mild reaction conditions with pH optima mostly between 6 and 8, are easy to handle, do not need expensive cofactors, are quite stable, and relatively simple in their molecular architecture in most cases. These "native" properties already make proteases useful synthetic tools for side-directed peptide cleavages, regiospecific ester hydrolyses, or the kinetic resolution of racemates (cf. chapter IV). Deeper insights into the mechanism of protease catalysis are needed to explain why proteases can also be useful catalysts to accelerate the "reverse of hydrolysis", which currently represents an important field of synthetic application of these enzymes. As catalysts, true to the definition familiar in chemistry, proteases alter the rate in which the thermodynamic equilibrium of the reaction is reached, but do not change that equilibrium itself. This inevitably implies that these enzymes work reversibly in both directions of the reaction. The equilibrium constants for the reverse reaction, however, are in the range of 10^{-3} to 10^{-4} L/mol.[8] Thus, under physiological conditions, the equilibrium position of the reaction is far over in the direction of hydrolysis while the reverse of hydrolysis indeed appears to be negligible in vivo. As a consequence, the use of proteases to catalyze the reverse of hydrolysis essentially needs manipulations to shift the equilibrium of the reaction. In practice, two basic strategies are considered to be synthetically useful: (i) the kinetically and (ii) the equilibrium-controlled approach. The rational behind the two approaches are already extensively reviewed by several authors.[7]

Scheme 2. Kinetic Model of Kinetically Controlled Synthesis Catalyzed by Serine and Cysteine Proteases[a]



[a] EH, free enzyme; Ac-X, substrate; [E..Ac-X], Michaelis−Menten complex; HX, leaving group; Ac-E, acyl enzyme intermediate; HN, acyl acceptor (nucleophile); Ac−N, synthesis product; Ac-OH, hydrolysis product.

Therefore, only a very short introduction to their fundamental basics are given in the following.

As already indicated by its designation, the equilibrium- or thermodynamic-controlled approach represents the direct reversal of proteolysis. Characteristic hallmarks of this approach are the use of acyl donors with free carboxylate function and the possibility of applying all proteases independently of their individual mechanism as potential catalysts. General drawbacks are the low reaction rates, the high enzyme requirement, and the need for direct approaches to shift the unfavorable equilibrium position. In the latter instance, manipulations to influence the ionization equilibrium are the method of choice. The rationale behind it is based on the thermodynamic barrier to the reverse of hydrolysis that is predominantly determined by the energy required for the transfer of a proton from the reacting group of the nucleophile to that of the negatively charged carboxylate moiety of the acyl donor (Scheme 1). Addition of organic solvents which lowers the dielectric constant of the medium finally resulting in reduced acidity of the acyl donor's carboxylate function increases the equilibrium constant for this proton transfer (K_{ion}) and, thus, promotes the reverse reaction. Furthermore, reaction conditions that lead to product precipitation or extraction increase the efficiency of the reverse reaction.

On the contrary, kinetically controlled syntheses only proceed successfully with serine and cysteine proteases that characteristically form reactive acyl enzyme intermediates during catalysis. A further hallmark of this approach is the use of slightly activated acyl moieties, such as esters or activated amides, as the donor components that significantly accelerate the rate of reaction and minimize the enzyme requirement. Initially, those acyl donors bind to the protease leading to a tetrahedral enzyme−substrate complex, which collapses to the covalent acyl enzyme intermediate Ac-E (Scheme 2). Activated carboxylate moieties are essential for this function while free carboxylates usually do not react with the enzyme under kinetically controlled conditions. The value of the kinetic approach for organic synthesis is based on the fact that Ac-E can be intercepted not only by water, but also by alternative nucleophiles

(HN), such as amines, alcohols, and thiols, that compete with water for deacylation. Kinetic control in this context means that the product appearing with the highest rate and disappearing with the lowest velocity would accumulate. Whereas the equilibrium-controlled approach ends with a true equilibrium, in the kinetic approach one is really only building up an intermediate product going through a maximum before the slower hydrolysis of the product starts to become important. Subsequently, the product will be hydrolyzed if the reaction is not terminated after the acyl donor is consumed and true equilibrium is allowed to be reached. From a chemical point of view, proteases that act in this kinetic mode can be seen as mild and selective reagents that are able to activate a generic carboxylic acid ester or amide moiety and transfer it to huge number of nucleophiles. The success of syntheses depends on several factors, while fundamental reaction parameters, such as temperature, ionic strength, reactant concentrations, and pH, play an important role. As a general rule, it can be considered that an increase of the nucleophile concentration (HN) and the pH also increases the product yield. The rationale is that both manipulations increase the efficiency of nucleophilic attack of the acyl enzyme intermediate by the acyl acceptor (HN) while the latter results from the fact that only the free-base form of the nucleophile can react with the acyl enzyme. For this reason, the pH of the reaction mixture should preferably be higher than the p*K* of the nucleophile. Finally, the enzyme itself significantly affects the efficiency of synthesis. Whereas the reaction rate is determined mainly by the specificity of the enzyme toward the acyl donor, a specific binding of the nucleophile to the S′ subsite of the protease is crucial for high yields (subsite notation according to ref 9). Since the specificity of the acyl donor and acceptor binding domains are individual parameters for each enzyme, the efficiency of synthesis and, thus, the synthetic utility of proteases for organic synthesis differs from one enzyme to the other.

III. Optimization of Proteases for Synthesis: Synopses of Current Techniques

It is easy to recognize that even with the most efficient enzyme and under kinetically controlled conditions proteases cannot act a priori as universal and perfect synthesis catalysts. Several serious drawbacks remain that are mainly: (i) forming of at least two synthesis products, i.e., the desired one and the hydrolyzed acyl donor, based on the competitive deacylation of the acyl enzyme by water. (ii) Most important, the specificities and selectivities of the available proteases seriously limit their synthetic application and, thus, do not enable all desired products to be assembled. As a consequence, only reactions with compounds closely related to preferred amino acid residues are of practical relevance, while nonproteinogenic amino acid moieties are not usually acceptable substrates of these enzymes. (iii) Particular in syntheses with longer peptides, there is a permanent risk of proteolytic side reactions of both the starting compounds and the products formed. (iv) Solvents, additives, and reaction conditions, e.g., pH and temperature, can strongly affect the enzyme's activity and stability. Summarizing these characteristics, proteases are far away from being perfect tools for catalyzing a broad spectrum of organic syntheses especially those that are based on the reverse activity of the enzyme. Further efforts are of decisive importance to overcome this limitation, in fact, to suppress competitive acyl donor hydrolysis, to alter the enzyme specificity and selectivity, and to suppress undesired proteolytic side reactions. A great number of recent publications in the field under reviewing reports on novel strategies or improvements of already existing ones useful to fit the enzyme properties to the requirements of synthesis are to be discussed within the following sections.

A. Medium-Engineering

The term "medium engineering" originally stems from Klibanov and co-workers[10] and refers to the possibility of influencing enzyme properties by altering the nature of the solvent in which the reaction is carried out. Historically, these studies have been mainly focused on investigations on the behavior of proteases toward organic solvents, first used as cosolvents and later as pure solvents practically without any water content. However, it should be remembered that beyond organic solvents there are other approaches useful for manipulating enzyme properties. Although less popular, at least when the number of publications is considered to be the only criterion, these include studies in frozen aqueous media, in frozen and supercooled organic solutions, in supercritical fluid extractions such as compressed carbon dioxide or propane, and in solid-to-solid reaction systems. Moreover, there are accounts that report on the combined use of those approaches. Essential backgrounds, trends, and the latest achievements in the field of medium engineering related to proteases shall be described in the following four sections.

1. Synthesis in Aqueous–Organic Mixtures

At first sight, the exchange of water with organic solvents breaks with the idea of "green chemistry" usually connected with the application of enzymes. However, the use of aqueous−organic mixtures instead of pure water as the reaction media originally had simple practical reasons. The major reasons are the frequently low solubility of the mostly hydrophibic reactants in pure water and the rise of unwanted spontaneous and enzymatic hydrolysis reactions. Note that for equilibrium-controlled synthesis the use of organic solvents is usually a need to shift the equilibrium to the formation of the desired product (cf. chapter II). Thus, the use of homogeneous aqueous−organic mixtures with highly solvating organic media such as DMF, DMSO, acetonitrile, or MeOH in both small or moderate amounts is a preferred approach in the kinetically and equilibrium-controlled synthesis. Recent examples are the synthesis of Z-Ala−Phe-NH_2 by pseudolysin in 35% MeOH,[11] the synthesis of a calcium mimic octapeptide in 10% acetonitrile,[12] or

the coupling of Boc-(D,L)-Ala-OMe and Boc-(D,L)-Tyr-OEt to H-Gly-NHNHPh using α-chymotrypsin and papain in 27% MeOH.[13] A large number of further examples are known from the scientific literature, and selections of recent ones will be presented in chapter IV. The additional effect of reducing unwanted hydrolysis of reactants and products is in the first instance directly connected with the decrease in water activity by the organic solvent, but usually needs a higher content of the organic solvent to be active. The rationale behind this is that the exchange of water with inert organic solvents decreases water concentration (water activity) and, thus, decreases the rate of competitive hydrolysis reactions. Recent X-ray structural investigations of subtilisin have been revealed that changes in the enzyme conformation also contribute to this effect. It was found that the active-site His residue flips in 50% DMF and the strong hydrogen bond observed between His and Asp in water is disrupted.[14] This finding could be confirmed by nuclear magnetic resonance, and provides a further mechanistic rationale for the decreased rate of undesired hydrolysis in the DMF−water cosolvent. This effect, however, is accompanied by others that influence the activity, stability, substrate specificity, and enantioselectivity of the enzyme. Unfortunately, not all properties of the enzyme take advantage from the organic solvent. Generally, the catalytic activity of proteases drastically decreases with increasing content of organic solvents. For example, the threshold DMF concentration (organic solvent concentration at which enzyme loses 50% of its initial activity in water) for chymotrypsin-catalyzed hydrolysis of the highly specific substrate Bz-Tyr-pNA was found to be only 26%.[15] The effect of diminishing enzyme activity by the organic solvent is not really surprising taking into account that proteases are usually designed by nature to be active in aqueous environments. Reducing the flexibility of the enzyme and its lower stability mediated by the denaturing effect of organic solvents are probably the major molecular reasons for this behavior.[16] In addition, the specificity and, thus, the specific activity of some proteases is closely connected with "catalytically active" water. The serine protease trypsin for example uses a water molecule that mediates highly specific binding of the amino group of lysine side-chains to Ser^{190} of the active-site of the enzyme.[17] To compensate for this undesired effect, the application of biphasic aqueous/organic systems (water/water-nonmiscible organic solvents) has been introduced 20 years ago.[18,19] In such systems, the enzyme is located in the aqueous phase while the reactants are dissolved either in the aqueous or the organic phase. Independent of the partition of the reactants in the two phases, catalysis takes place in the aqueous phase rather than at the interphase between the two solvents. In every case, the reaction is promoted by product extraction into the organic phase that prevents secondary cleavage of the product formed and shifts the equilibrium position to the side of the product in equilibrium-controlled syntheses. The synthetic utility of this approach has been successfully demonstrated for a set of various proteases as well as a number of synthesis reactions and is already reviewed in detail.[20] However, several drawbacks remain, whereas the major drawback is the limitation of the approach to hydrophobic products with low water solubility. Additionally, prolonged reaction times may occur due to the additional partition equilibria. Intensive stirring usually used to enhance the rate of mass transfer across the interphase, however, drives the rate of enzyme denaturation and inactivation due to interfacial tension.[21] A more recent study reports on the equilibrium-controlled synthesis of the protected dipeptide Boc-Gly−Phe-OMe from Boc-Gly-OH and H-Phe-OMe using papain as the catalyst and various biphasic systems.[22] With regard to a former study,[23] the authors investigated the influence of the mode of stirring and the nature of the organic solvent on the stability of the enzyme. As indicated by Table 1 in both cases a more or less fast inactivation was found. A rationale for the distinct effects of the organic solvents was found by the proportionality between the droplet size of the organic solvent suspended in the aqueous phase and the enzyme stability. This reveals that a large interfacial area between water and the organic solvents accelerates inactivation. Surfactants such as Tween 80 that accumulate at the interphase reduce the interfacial tension and lead to prevention of enzyme activity—an effect that seems to be not restricted to proteases but also holds for lipases.[24] Recent mechanistic studies using papain as the catalyst led to a model that allows the prediction of the enzyme stability in such biphasic systems with a correlation coefficient of 0.829 between the model and the experimental data.[25] From the same laboratory, an approach has been developed for predicting changes of the aqueous phase pH on the basis of the partition of reaction substrates in biphasic systems which has been shown to be useful for selecting optimum reaction conditions.[26] Further recently published papers document the successful application of such biphasic systems for other proteases such as cardosins A and B[27] or thermolysin,[28] but also for other enzymes such as amine oxidases,[29] immobilized beta-glycosidase,[30] chloroperoxidase,[31] P-galactosidase from *Aspergillus oryzae*,[32] penicillin acylase,[33] and many others.

Table 1. Effect of Stirring and the Nature of the Organic Solvent on the Stability of Papain in Biphasic Systems[a]

	half-life (h)	complete inactivation (h)
standard aqueous conditions	>50	>50
stirring (600 rpm)	32	42
biphasic conditions with (shaking bath):		
carbon tetrachloride	2	5
trichloroethylene	3	8
cyclopentanone	n.t.	15
benzene	9	20
toluene	9	20
n-heptane	n.t.	>25
trichloroethylene + 0.4% Tween 80	n.t.	>50

[a] According to ref 21 with permission from Elsevier Science. Copyright 1988. n.t., not tested.

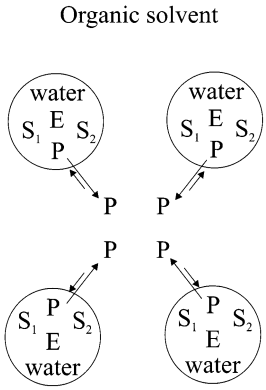


Figure 1. Schematic architecture of reverse micellar systems. E, enzyme; $S_{1,2}$, substrates; P, product.

As mention above, the extraction of the synthesis product into the organic phase is an essential condition for using biphasic systems. Thus, one could assume that this approach is preferred to the synthesis of hydrophobic compounds and therefore may be limited in its synthetic utility. This assumption, however, was shown to be partly wrong. Recent studies have been demonstrated the usefulness of ternary biphasic systems consisting of surfactants, water nonmiscible organic solvents, and small amounts of water to the synthesis of hydrophilic peptides, such as Z-Arg−Gly-NH_2, Z-Gly−Asp-NH_2,[34] and closely related peptides,[35] by various serine and cysteine proteases. The formation of reverse micelles in such low-water containing biphasic systems provides a rationale for this activity. These colloidal systems are formed spontaneously upon addition of a small amount of water to a large volume of water nonmiscible organic solvents containing a surfactant agent. "Reverse" in this context means that in contrast to normal micelles the hydrophilic ends of the surfactant are turned outside into the surrounding organic solvent forming spherical aggregates with 1−10 nm diameter that encapsulate the protease inside into an aqueous micro-milieu. Although the properties of the micellar water differ markedly from those of bulk water, proteases entrapped in reverse micelles usually exhibit very high or so-called "super activity".[36] From a synthetic point of view, reverse micelles can be considered as microreactors that are separated from the organic solvent by a surfactant layer as illustrated by Figure 1. The first synthetic use of such reverse micellar systems dates back to 1978 and was mainly pioneered by Martinek et al.,[37] Luisi,[38,39] Menger and Yamada,[40] and the group of Morihara.[41] These groups could show that reverse micelles not only facilitates the synthesis of moderate and bad water soluble peptides, but also of those starting from components that are almost insoluble in water. Besides the finding that reverse micelles enable both the kinetically and equilibrium-controlled synthesis of hydrophilic peptides, recent studies[33,42] include investigations on the influence of the water content, temperature, pH, surfactant, organic solvent, and protease on the course of peptide synthesis. It was summarized that the water content and especially the ratio between the water and the surfactant content, known as W_0 ($W_0 = [H_2O/[\text{surfactant}]$), is the most noticeable parameter affecting on the one hand the physical characters of the micelles, e.g., sphere size and micelle stability. On the other hand, the amount of water directly affects the enzymatic reaction by influencing the enzyme activity, the enzyme and reactant solubility, and the extent of hydrolytic side reactions. Temperature and pH seem to affect the enzymatic reaction in a rather general way as it is known for other systems. Some additional influence of the pH on the partition of the reactants and products, especially those with charged functionalities, and the stability of the reverse micelles could be observed. A strong influence has been found for the surfactant and the organic solvent and, thus, of the nature of the reverse micellar system. While reactions with the bis(2-ethylhexyl)sodium sulfosuccinate (AOT)/isooctane system led to about 80% yield in trypsin-catalyzed synthesis of Ac-Gly−Asp(OMe)-OMe the use of other reverse micellar systems such as hexadecyltrimethylammonium bromide (HTAB)/heptanol/hexanol and Triton X-100/ethyl acetate significantly decreased the efficiency of syntheses or even hindered product formation.[42] In another recently published study, the stability of proteases encapsulated in reverse micelles has been investigated in comparison to aqueous conditions and various homogeneous water−organic mixtures using chymotrypsin as the model enzyme.[43] Former studies gave inconsistent results that either reported a higher[37] or lower[44] enzyme stability in micellar systems than in aqueous solution. The recent study considered different system parameters to be the reason for this discrepancy and has been demonstrated a high stability of enzymes encapsulated into reverse micelles, being even better than the water−organic mixtures and also the aqueous medium. Additionally, it has been shown that long chain alcohols, such as hexanol, octanol, or decanol, used as cosurfactants can further stabilize the encapsulated enzyme. Furthermore, it was found that the presence of such aliphatic alcohols increases the solubility of polar reactants in the organic solvent.[45] For synthetic use, however, it must be considered that those alcohols, which may act as competitive nucleophiles, can promote side reactions such as transesterification or esterification in kinetically and equilibrium-controlled synthesis, respectively.[40,46] In summary, it can be concluded that the use of the reverse micellar system prevents enzyme inactivation in organic solvent and allows for the synthesis of both hydrophobic and hydrophilic compounds. Remaining drawbacks for a simple practical use are the lack of rational methods to optimize these multiple component systems. In addition, the presence of surfactants in the reaction system makes separation and purification of products often difficult. These problems still limit the broad application of reverse micelles in enzymatic synthesis.

An alternative to conventional biphasic and micellar systems has been recently published by Clapes et al.[47] The researchers reported on the use of water-in-oil (W/O) gel emulsions as novel reaction media for chymotrypsin-catalyzed kinetically controlled peptide synthesis. W/O gel emulsions are liquid−liquid colloidal systems with low oil and surfactant concen-

trations (<5%) and large amounts of water (>95%)—a composition exactly opposite to micellar systems. The architecture of such emulsions consists of close-packed water droplets with radii typically of a few microns, separated by a thin film of continuous phase. W/O gel emulsions can solubilize large quantities of hydrophilic and hydrophobic compounds as well. In fact, an emulsion containing 95% water is as effective as 40% DMF in water while retaining a high catalytic enzyme activity. No evidence to mass transfer limitations has been found. Although such reaction systems are occasionally used in organic synthesis,[48] their application in biocatalysis is still at the preliminary stage. So it is unclear whether W/O gel emulsions are equally useful to suppress unwanted water-mediated side reactions.

2. Synthesis in Neat Organic Solvents

Because undesired hydrolysis and substrate solubility in aqueous media are one of the main drawbacks, the use of neat organic solvents should be the ultimate way to improve the utility of proteases for organic synthesis. Indeed, this approach has proven to be highly useful expanding the range and efficiency of practical applications of these enzymes. Reactions that are usually impossible in aqueous media, e.g., esterification, transesterification, or reactions on originally nonspecific (nonnatural) water sensitive acyl and nucleophile moieties, often proceed in organic solvents with high efficiency.[49] It is not surprising, therefore, that a remarkable number of academic and industrial laboratories worldwide increasingly turn their attention to nonaqueous biocatalysis and, thus, tremendous progress has been made in recent years. A substantial number of actual reviews document this impressive development highlighting important aspects of nonaqueous biocatalysis in detail. For example, Klibanov[50] reviewed general effects of organic solvents on the enzyme activity, stability, and specificity; Khmelnitsky and Rich[51] highlighted approaches to the improvement of the enzyme activity in organic solvents; aspects to the modeling of enzyme properties in organic solvents have been discussed by Colombo et al.[52]; Halling[53] reviewed the function of counterions and acid−base effects on the biocatalysis in low-water media; and Carrea and Riva[54] published a comprehensive review covering basic effects of neat organic solvents on the enzyme properties as well as presented recent examples for the synthetic application of this approach. To avoid redundance, discussion of this field, although exciting, is therefore consciously reduced to the main important background and the major trends of the last years strongly related to proteases. Guidance through the section is aided by a kind of subdivision indicated by *keywords* into effects of the organic solvent on the enzyme stability, conformation, specificity, activity, and approaches to improve proteases for acting in neat organic solvents.

Straightforward extrapolation of the denaturing effect of increasing concentrations of organic solvents, as it is known from homogeneous aqueous−organic mixtures,[55] indicates a dramatic decrease in *enzyme stability* due to a fast enzyme unfolding in neat organic media. This assumption, however, has been shown to be wrong. In fact, proteases and also other enzymes, such as ribonuclease,[56] porcine pancreatic lipase,[57] terpene cyclase,[58] or ATPase and cytochrome oxidase,[59,60] are more stable in neat organic solvents than in aqueous−organic mixtures even when compared to water. For example, chymotrypsin remained active for several hours when incubated at 100 °C in anhydrous solvents, whereas in water it deactivates within seconds at this temperature. The rationale behind this is that water acts as a molecular lubricant promoting the conformational flexibility essential for enzyme unfolding. In anhydrous organic solvents, however, enzymes are very rigid and, as a result, are kinetically fixed in their prior conformation. Consequently, it is now usually recognized that not the organic solvent itself stabilizes the enzyme molecule toward denaturation, but the absence of water although the drive to unfold is even greater in anhydrous organic solvents than in aqueous−organic mixtures.

Similarly, the effect of organic solvents on the *enzyme conformation* is usually much lesser than originally thought. In the meantime, it became a subject matter that very little "essential" water (approximately 0.3 to about 1%) is actually required for proteins to maintain their catalytic active conformation. Recent studies impressively proved this fact for slightly cross-linked subtilisin dissolved in anhydrous dioxan by X-ray crystallography.[61] Cross-linking of the enzyme, although it represents an artificial modification, was chosen to make subtilisin soluble in dioxan which is essential for crystallization. Despite using dry dioxan and lyophilized subtilisin, several water layers around the protein molecule were found in the X-ray structure. Comparison of the resulting crystal structure with that in water has been shown that both the enzyme's overall structures and the structures of the active-sites are virtually identical. The same holds true for the structure found in dry acetonitrile[62] indicating that neither the organic solvent itself nor its nature influences the crystal structure of the protease. A comparable picture has been found for another protease, i.e., chymotrypsin, analyzing the X-ray structures of the enzyme dissolved either in hexane or water.[63] Besides X-ray crystallography, these findings have been further proved by Fourier transform infrared (FTIR) spectroscopy using subtilisin suspended in a large number of organic solvents, such as octane, hexane, tetrahydrofuran, or ethyl ether.[64] Similarly, it was found that the enzyme's secondary structure by means of the α-helix and β-sheet contents is fairly insensitive to the nature of the organic solvent differing widely in their physicochemical properties. Surprisingly, a markedly different situation has been found when organic solvents were used in which lyophilized subtilisin is soluble instead of being suspended (Figure 2).[65] Calculation of the α-helix and β-sheet contents from the FTIR spectra reveals that only glycerol restores the secondary structure to its native aqueous level (Table 2). On the contrary, all other organic solvents tested lead to significant changes in the secondary structure.

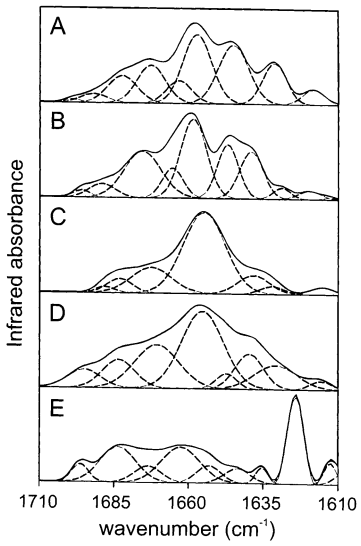


Figure 2. Fourier transform infrared (FTIR) spectra of subtilisin dissolved in (A) aqueous buffer solution, (B) glycerol, (C) 2,2,2-trifluoroethanol, (D) trichloroethanol, and (E) DMSO (according to ref 65 with permission from John Wiley & Sons Inc. Copyright 1997). (Solid lines) resolution enhanced spectra, (dashed lines) Gaussian bands. The enzyme concentrations were as follows: (A) 40 mg/mL, (B) 10 mg/mL, (C) 10 mg/mL, (D) 5 mg/mL, (E) 10 mg/mL.

Table 2. Secondary Structure of Lyophilized Subtilisin Dissolved in Various Solvents and in the Dry Form[a]

solvent	α-helix content (%)	β-sheet content (%)
water (pH 7.8)	34 ± 0	19 ± 0
lyophilized subtilisin powder	26 ± 2	29 ± 2
glycerol	38 ± 2	20 ± 5
2,2,2-trifluoroethanol	60 ± 2	20 ± 2
2,2,2-trifluoroethanol	34 ± 3	29 ± 3
DMSO	6 ± 0	5 ± 2[b]

[a] According to ref 65 with permission from John Wiley & Sons Inc. Copyright 1997. [b] Additionally, 27 ± 3% of denatured β-sheets was detected in this solvent.

Parallel kinetic studies could show that these changes are strongly related to a decrease in enzyme activity in a proportional manner. These findings reveal that enzyme suspensions and dissolutions in anhydrous organic media influence the structure of proteases in a fundamental different manner, a fact being of decisive importance for their synthetic use.

One could expect that the similar conformation of enzymes in water and in neat organic solvents should guarantee for similar substrate *specificities*. Nature draws a completely different picture, however. In fact, it has been found that the substrate specificity including enantiomeric, prochiral, regio-, and chemoselectivities can change profoundly on switching from one solvent to another.[10,66,67] Due to the importance of this phenomenon for synthesis, it is not surprising that a number of researchers turned their attention to rationalize the basic reasons for this effect on a molecular level. For example, Bross et al.[68] correlated the increased enantioselectivity of subtilisin with increasing flexibility of the enzyme pointing out that both are directly related. Although an opposite behavior could be expected from classical "rules" of enzyme enantioselectivity,[69] the model has been proved successfully to predict the enantioselectivity of the enzyme in a number of different organic solvents. In a later work, Rariy and Klibanov contradict to this model postulating that the increase in subtilisin's enantioselectivity occurs despite the increase in enzyme flexibility but is promoted by other more impactful factors, such as hydrophobicity and dielectric constant.[70] Several further papers have been published from Klibanov's group describing a model that correlates changes in enantioselectivity with differences in substrate solvation in the enzyme−substrate complex.[71] It has been found that the approach worked well for cross-linked crystals of subtilisin and chymotrypsin, but not for enzyme suspensions and enzymes in aqueous−organic mixtures.[72] Colombo et al. reported on the use of Klibanov's method to rationalize results found for subtilisin-catalyzed transesterifiaction reactions but only found a weak correlation. Possible reasons why this method fails sometimes are summarized in a review of Halling.[73] He reminds us that solvation free energies, which are the basic correlation parameters of this method, are not necessarily expected to be group additive because of the importance of entropic contributions. Overbeeke et al. studied the selectivity of lipase-catalyzed hydrolysis in water and esterification in hexane and found a contribution of enthalpy and entropy to enzymatic enantioselectivity which may be also useful for proteases.[74] However, further studies, in particular the estimation of enthalpies and entropies, are essential to evaluate this approach. There is no doubt that all these models are useful to predict enzyme enantioselectivities in organic solvents in a more or less exact manner. However, none of them is likely to be generally applicable, which may reflect that solvent effects on enzyme selectivities are not caused by a single mechanism.

Generally, despite adopting the right conformation of proteases suspended in neat organic solvents, their *catalytic activities* are far lower than in water or aqueous−organic mixtures. For chymotrypsin and subtilisin for example, Zaks and Klibanov found 10^4−10^5-times lower activities of the two enzymes in anhydrous octane than in water while the exchange of octane with other organic solvents even led to a further decrease in activities of about 2 to 3 orders of magnitude.[75] In a recent review, Klibanov[76] marked this serious drawback "as the only dark cloud hanging over nonaqueous enzymology" while Gill et al.[77] also pointed out that the toxicity of numerous organic solvents may at least partly limit the synthetic utility of organic media especially for application in the food sector. In fact, the dramatic decrease in enzyme activity seriously threatens the synthetic utility of nonaqueous catalysis especially for larger-scale synthesis. Addressing the reasons for the low activity of enzymes suspended in anhydrous media is, therefore, a challenging task of outstanding interest being presently not completely solved. Actually, a set of several factors that may act in an additive manner are considered to be important for this effect.[57] The insolubility of enzymes in nearly all commonly used organic solvents, which could be an obvious explana-

tion, may be one of them. In this context, mainly diffusional limitations on the substrates caused by the enzyme insolubility are discussed. Presently, there is no general agreement that holds for all enzymes and enzyme forms. While an internal mass transfer limitation was found for enzymes with high activity and large particle size[78] or immobilized enzymes with high loading,[79] this was not the case for subtilisin suspended in several organic solvents.[80] A general and by far larger effect on the enzyme activity is discussed based on the influence of the organic solvent on the activation energy of the reaction. At least two factors are expected that directly increase the activation barrier relative to water. One focuses on unfavorable energetics of substrate desolvation in organic solvents (especially for hydrophobic substrates)[81] and the other focuses on the lower capability of organic solvents to stabilize the charged tetrahedral substrate−enzyme intermediate.[10] To a similar extent, the reduced conformational mobility connected with a highly rigid structure of proteins in anhydrous solvents is considered to diminish the catalytic power.[82,83] Especially hydrophilic organic solvents, i.e., water-miscible solvents, reinforce this rigidness of the enzyme due to their strong effort to strip the essential water tightly bound to the enzyme molecule, an effect that has been rationalized by Halling[73,84] and Goderis et al.[85] in terms of the thermodynamic water activity (a_w). Further decrease of enzyme activity can result from an effect which is known as "pH memory" of the enzyme.[46] The rationale is that the protonation state of an enzyme in organic solvents, which controls for the enzyme activity, reflects the pH of the last aqueous solution to which it was exposed, a phenomenon that could directly and quantitatively elucidated on the basis of recent FTIR spectroscopy studies.[86] Accordingly, maximal enzyme activity can only be reached if the pH of this solution, which is usually that from which the enzyme was lyophilized or precipitated, corresponds to the pH optimum of the enzyme. It must be noted, however, that the formation of acidic or basic products during the reaction can alter the protonation state and, thus, the activity of the enzyme. Finally, the lyophilization process itself can change the structure of the enzyme (cf. Table 2) and, therefore, can decrease the enzyme activity due to the rigidity of enzymes in organic solvents that prevents refolding into the catalytically active conformation. On the contrary, competitive binding of the organic solvent in the active-site of the enzyme or conformational changes caused by direct contact of the enzyme with the solvent appear to be of lesser responsibility for activity reduction.

In summary, neat organic synthesis media have numerous interesting advantages over water and aqueous−organic mixtures which, however, are accompanied by a similar number of drawbacks. One of the most serious is certainly the dramatic activity reduction. This becomes even more important since the requirement for a solvent in which enzyme activity and stability are optimal and the substrates show high solubility are often conflicting needs. Thus, hydrophobic solvents that are regarded as most suitable for the application of enzymes do not usually solubilize commonly employed reactants. On the contrary, the influence of organic solvents on the enzyme specificity and selectivity can be considered both as an advantage and a disadvantage. Although these effects are usually less than a factor of 10 and, thus, small in energy terms, they are often large enough to change the course of synthesis in a useful fashion. The general prediction of such changes, however, is presently still limited as described above. As a result, time-consuming experimental efforts are needed to optimize the conditions for each single synthesis reaction.

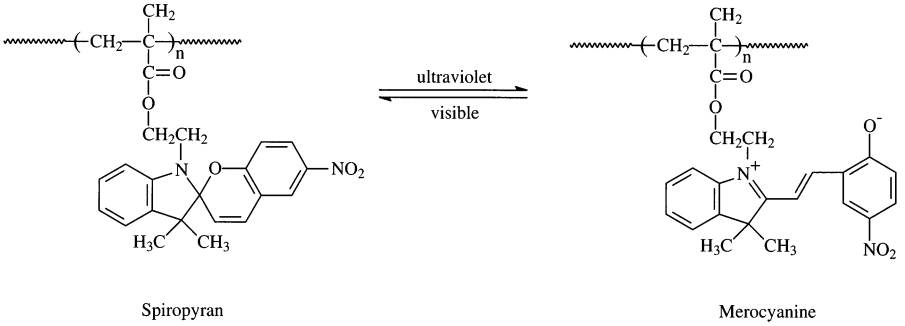
3. Improving Proteases for Synthesis in Organic Solvents

A large number of papers were published in the period of reviewing which deal with the use of proteases in nonaqueous organic solvents report on techniques to improve the enzyme's catalytic activity. Recognizing this fact and its importance for the general synthetic application of proteases in such media certainly justify discussion of the latest efforts in its own section. Generally, the techniques described in the literature focus either on the engineering of the organic solvent or, in most cases, on manipulations on the enzyme formulations. Genetic as well as site-specific chemical approaches to adopt proteases for catalysis in organic media are initially excluded and will be discussed separately in section C.

The individual effect of organic solvents on the enzyme activity has been already discussed partly in the foregoing section. Besides hydrophobic solvents, the main improvements can be reached when solvents with water-like physicochemical properties are used in which the enzyme is not suspended but dissolved. In particular, *water mimics*, such as glycerol (cf. Table 2), restore enzyme activities in the most efficient manner. However, going back to water-like solvents usually destroys the advantages of neat organic media leading to limitations similar to that of pure water systems. Glycerol, for example, while mediating an activity almost identical to water,[65] simultaneously acts as a competitive nucleophile and, thus, leads to water-like side reactions.

Dissolution of the enzyme in anhydrous solvents can be also reached by *chemical modification* of the enzyme avoiding the occurrence of solvent-mediated undesired reactions. Such modifications range from simple nonspecific acetylation of the enzyme's free amino groups[87] over the modification with hydrophilic moieties such as carbohydrate-polyacrylate polymers[88] to the linkage of amphipathic compounds to the enzyme surface.[89] For example, covalent attachment of poly(ethylene glycol) (PEG)[90] or of the amino group-reactive methoxypoly(ethylene glycol) (MPEG),[91] which represent commonly used amphipatic polymers, mediate enzyme solubility both in aqueous and numerous organic solvents. The effect of PEG-modification on the enzyme activities, however, is different in the two media. While in water the enzyme activities are partially lost, an opposite behavior was found for the organic solvent system. Further studies have been shown that the hydrated

Scheme 3. Photoisomerization of Spiropyran in Response to UV Light



Spiropyran Merocyanine

PEG chains create an aqueous shell around the enzyme molecule that stabilizes the catalytic active conformation and, thus, promotes the catalytic activity of the enzyme in anhydrous solvents.[92] Recent investigations could show that PEG-subtilisin adopts identical conformations in water and dioxan as evidenced by circular dichroism and intrinsic protein fluorescence measurements.[93] Stabilization of the active enzyme conformation however was accompanied by a reduced stability and an increased drive to autolysis in organic solvents than the corresponding enzyme powder.[93] Similar studies on MPEG-modified proteases, such as trypsin,[94] subtilisin,[95] and chymotrypsin,[96] contradict this finding on the reduced stability of these modified enzymes. Attempts have been done to rationalize these diverse effects on enzyme stability. A recent one, which combines kinetic analysis of enzyme inactivation experiments with molecular modeling computation, reveals an increased number of hydrogen bonds for MPEG-modified trypsin that was postulated to be the reason for the increase in enzyme stability.[97] Additionally, the number of polymer-modified amino acid sidechains of the enzyme may account for the differences in stability observed.

An exiting approach was published by Ito et al. which can be certainly considered as a highlight in the field of polymer-modified enzymes.[98] The authors synthesized a hybrid subtilisin the solubility of which can be regulated by photoirradiation through coupling with a photoresponsive polymer. Although some methods of chemical modification for regulating enzyme solubility in aqueous media have been already reported, such as attachment of pH-,[99] ionic strength-,[100] temperature-,[101] or redox-sensitive[102] polymers, that was the first approach acting in organic solvents. The polymer characteristically contains photoresponsive spiropyran groups that allow direct manipulation of the solubility of the modified subtilisin as the nonpolar spiropyran group is reversibly converted to the polar merocyanine form by UV and visible light irradiations (Scheme 3). The spiropyran−polymer−subtilisin complex was well-soluble in toluene and chloroform and catalyzed transesterification more than 100 times faster than the free enzyme. After ultraviolet irradiation, the modified subtilisin precipitated and could be easily and quantitatively recovered by simple centrifugation. The recovered enzyme, resolubilized by visible light irradiation, retained its initial activity even after several cycles of precipitation and solubilization.

Improvements of the activity and solubility of proteases do not essentially need covalent enzyme modification, but can be also reached by *physical modifications* with lipids,[103] surfactants,[104] PEG, and other polymers.[105] Modifications of this type can be performed by simply lyophilizing enzymes in the presence of the appropriate additives. Particularly for surfactant-coated enzymes some parallels to reverse micellar systems could be expected (cf. section 1 of this chapter) although the molecular structure of surfactant- and also lipid-modified enzyme complexes is presently not clear. Generally, enzyme coating of this type leads to a significant increase in enzyme activity in organic solvents which usually exceeds that of chemically modified enzymes. Complexation of subtilisin and chymotrypsin with the surfactant bis(2-ethylhexyl)sodium sulfonate (Aerosol OT) for example, mediates complete dissolvation of the enzyme in isooctane and increases the catalytic efficiency to be only 2-fold less compared to that found in aqueous media.[106] Interestingly, no hints to the formation of reverse micelles could be found. Within subtilisin-catalyzed reactions using dioleyl *N*-D-glucono-L-glutamate as the surfactant Okazaki et al. could show that the addition of extraneous water did not further increase the rate of reaction, but promotes hydrolytic side reactions.[107]

In another report, Vakurov et al. investigated the properties of chymotrypsin−polyacrylate complexes used in low-water containing DMF and acetonitrile. Similarly, complexation retained the enzyme activity while the free enzyme already became inactive at 60% DMF.[108] The reason for the impressive rate of acceleration may be that adding a hydrophobic support before lyophilization not only stabilizes the enzyme structure, but may also allow the enzyme to adopt a more active conformation.[109] It should be noted however that Kahlaf et al.[110] found a similar activating effect of surfactants on cross-linked enzyme crystals which are expected to be highly rigid and, thus, should be unable to undergo significant conformational changes. Remaining drawbacks for a simple practical use of the approach mainly stem from the presents of the lipids and surfactants themselves that usually hinders the separation and recovery of products from the reaction mixture. In addition, the degree of enzyme activation was found to be dependent upon the chemical nature of the coating component,[111] while the lack of rational methods hinders its prediction.

Besides lipids, surfactants, and amphipathic polymers, a quite unexpected enzyme activation effect has been recently described in two independent studies for denaturing agents,[112] such as sodium dodecyl sulfate (SDS), urea, or guanidine hydrochloride, and denaturing solvents[113] such as dimethyl sulfoxide or formamide. The idea of using denaturing agents for protease-catalysis originally stems from the assumption that their presence in the reaction mixture may increase the solubility of highly hydrophobic peptide reactants in polar organic solvents and aqueous−organic mixtures. Simultaneously, a reduced enzyme activity was originally expected due to the well-know diminishing effect of denaturants on the enzyme structure in water. In organic solvents and aqueous−organic media, however, a completely different behavior has been found. In fact, pepsin-catalyzed ligations of a number of hydrophobic peptide fragments could be efficiently performed in the presence of 4.3 M urea.[112] Similarly, high reaction rates and efficiencies were found for solvents containing 6 M guanidine hydrochloride or 0.5% SDS. Furthermore, model reactions with subtilisin and thermolysin revealed that the activating effect caused by denaturants may be a general phenomenon. Very recently, Guo and Clark report on some restrictions of the approach, however.[114] While the promoting force of urea could be verified for subtilisin, a complete inactivation of chymotrypsin was found under the same conditions illustrating that the effect of denaturants is not completely general. Detailed studies on the catalytic behavior of SDS−subtilisin complexes have been published by Getun et al.[115] Clarification of the dependence of the activating effect of SDS on the nature of the organic solvent and the influence of the carboxyl activation on the course of reaction were the main important findings of these investigations.

Physical modifications of enzymes on inert solid matrixes, such as pore glass, polyamide, Celite, silica, various zeolites, polypropylene or alumina,[116] are usually termed as *noncovalent immobilization* (*adsorption*). Generally, the degree of individual enzyme activation and stabilization by adsorption is strictly dependent upon the nature of the solid matrix. Additionally, a strong influence of the ratio of enzyme/support on catalysis was found.[117] While at high loadings the enzyme activity becomes reduced due to diffusional limitations on the substrates, low loadings lead to unfavorable interactions of the catalyst with the matrix. The latter can be prevented by adding extraneous proteins that mask uncovered matrix surfaces responsible for enzyme inactivation.[118]

Besides solid matrixes, noncovalent enzyme immobilization can be achieved by entrapment of the catalyst in hydrogels, such as chitosan, carrageenan, cross-linked poly(vinyl alcohol), and calcium alginate.[119] Hydrogels are enzyme−polymer complexes in which the entrapped enzyme is retained inside the hydrated polymer coils without covalent binding. In a recent paper, Kuptsova et al. report on the use of poly(*N*-vinyl caprolactam)-Ca alginiate (PVCL-CaAlg) hydrogel for immobilization of trypsin and chymotrypsin.[120] PVCL-CaAlg is a temperature-sensitive polymer that spontaneously forms hydrogels upon a temperature increase over 37 °C with simultaneous entrapment of the enzymes. Each PVCL molecule contains at least 15 water molecules per polymer chain that provide a favorable microenvironment for the enzymes.[121] Mass transfer limitations on substrates are minimized by the Ca alginate additive which provides a macroporous structure with low diffusional constraints.[122] By using PVCL-CaAlg hydrogels, retention of 40% of the initial activities of trypsin and chymotrypsin in 90% DMF and 99.5% acetonitrile, respectively, could be reached.[120] Furthermore, the general possibility of repeated usage of PVCL-CaAlg entrapped proteases in cyclic processes has been demonstrated on the example of chymotrypsin and a total time range of 270 h.

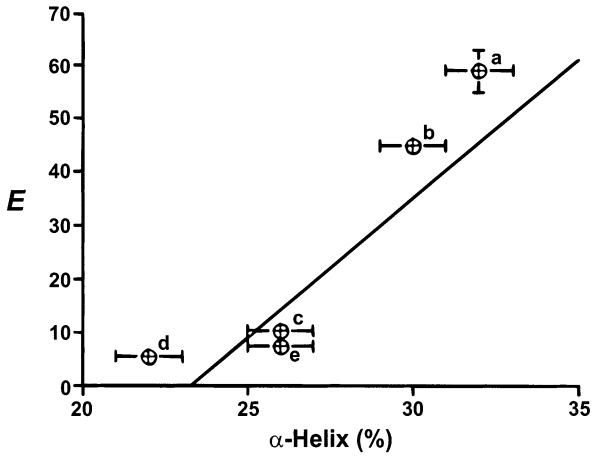


Figure 3. Dependence of the enzyme enantioselectivity on the α-helix content for the subtilisin-methyl-β-cyclodextrin formulation (according to ref 123 with permission from the American Chemical Society. Copyright 1999). Solvents: (a) THF, (b) 1,4-dioxan, (c) methylene chloride, (d) acetonitrile, and (e) toluene. The solvents with the highest enantioselectivity (a, b) also lead to the highest enzyme activity.

In another recent study, Griebenow et al. report on the use of methyl-β-cyclodextrin as immobilizing matrix for subtilisin.[123] Cyclodextrins are versatile macrocyclic compounds that can form inclusion complexes with a number of guest molecules and have been shown to be useful in a number of applications ranging from chiral separations to drug delivery and as enzyme mimics.[124] Preparation of subtilisin−cyclodextrin complexes can be achieved by simple colyophilization. Model transesterifications between *sec*-phenethyl alcohol and vinyl butyrate revealed an increase in reaction rates up to 164-fold and a 2-fold improvement of enantioselectivity for such complexes compared to the free enzyme. Addressing these effects by FTIR spectroscopy suggests that the cyclodextrin is partially efficient in ameliorating dehydration-induced structural perturbations and structural mobility of the enzyme. This function, however, was found to be dependent upon the organic solvent used. As shown in Figure 3 high enantioselectivity and activity can be only reached if the organic solvent retains the structural integrity of the enzyme. Pres-

ently, it is unknown whether the individual substrates may also contribute to similar effects on the efficiency of synthesis.

An interesting approach to polymer-entrapped enzyme catalysts has been published by Dordick and co-workers that is based on a novel type of biocatalytic material, so-called biocatalytic plastics.[125] These polymer−enzyme complexes, which are prepared directly in nonaqueous media by copolymerization of vinyl monomers with the enzyme, can contain up to 50% (w/w) total protein. Attempts to incorporate chymotrypsin and subtilisin led to highly active enzyme formulations in both aqueous and organic media enabling the synthesis of peptides, sugars, and nucleoside esters with high efficiencies. Long-time range stability studies on "chymotrypsin-plastics" have been shown that the enzymes retained full activity in hexane for three weeks and was nearly as active as the starting organic solvent-soluble enzyme.

Covalent immobilization of proteases on solid supports is well-known to result in highly stable catalysts mainly due to rigorous decrease in conformational mobility and, additionally, makes catalyst recovery very easy. Therefore, it is not surprising that covalently immobilized enzymes have found widespread application not only for synthesis, but also for the development of biosensors, immunoassays, and bioseparations[126] where high stability and long lifetimes are important needs. Generally, because enzymes are usually insoluble in most organic solvents there is no essential need to attach them covalently on the support, although increased enzyme stability has been observed in various organic media.[127] A number of recent studies published in the period under review add to a long list of former papers in this field and a short summary will be given in the following section.

In general, the immobilization methods currently used can be divided in two main strategies: (i) random immobilization and (ii) site-specific attachment of the enzyme to the solid support. Random immobilization reflects the fact that the enzyme's amino and carboxyl groups usually used as the anchor functionalities randomly reacts with their counterparts on the solid support. Thus, differences in the site of attachment inevitably lead to differences in the orientation of an enzyme on the support surface. If the site of attachment is close to the binding or active-site, the activity of the enzyme may be partially or totally lost due to steric hindrances. This is one of the reasons that an increase in stability of the enzyme by covalent immobilization is often accompanied by a decrease in enzyme activity. In addition, the nature of the carrier material itself and the type and conditions for the chemical reaction involved in the attachment procedure strongly influence the stability and activity of the immobilized enzyme.[128] Frequently used carrier materials for protease immobilization are for example controlled pore glasses,[129] agarose matrixes,[130] carboxymethylcellulose,[131] Chitosan beads,[132] or polyethylene-based graft polymers.[133] A number of industrially important chemicals are produced using immobilized proteases where the artificial sweetener aspartame is certainly one of the most famous.[134]

Decrease of enzyme activity due to nonproductive orientation of the biocatalyst on the support can be avoided by using site-directed attachment technologies. In general, two different approaches have been recently developed and both of them are based on genetic engineering to modify enzymes with unique functionalities that allow for controlled immobilization.[135] In one such approach, a protein molecule bearing a unique amino acid residue with a side-chain group can be site-directly immobilized. This method was used by Huang et al. for site-specific attachment of subtilisin to various carrier materials.[136] The authors used the fact that the wild-type subtilisin originally contains no cysteine residue. Thus, genetic introduction of a cysteine moiety leads to a unique thiol group that can be used as the site-specific anchor. On the basis of the three-dimensional structure of subtilisin the serine residues 145 and 249, respectively, were selected as potential mutation sites being away from the active-site of the enzyme. The resultant enzyme variants S145C and S249C have been finally immobilized on thiol-reactive thiol Sepharose 4B, thiopropyl Sepharose 6B, and Affi-Gel 501 organomercurical beads. Ordered two-dimensional arrays of enzyme molecules on the support surface were obtained with the active-sites of the enzyme oriented toward the solution phase. It was found that the site-directed immobilized subtilisin had higher catalytic activity and stability than randomly immobilized enzyme. Similar studies with thermolysin have been shown that controlled multiple fixations can increase the stability of the enzymes additionally.[137] In instances where the active-site is not close to the N- or C-terminus of the enzyme, an alternative approach based on the incorporation of affinity tags can be used. Attachment of the affinity label to the enzyme of interest can be achieved by a gene fusion approach. The resultant fusion protein conjugate can then be immobilized through the affinity tag on appropriately modified surfaces.[138] Recently, Wang and co-workers used this approach to the site-specific immobilization of subtilisin.[139] For this purpose, an octapeptide affinity tag, Asp−Tyr−Lys−Asp−Asp−Asp−Asp−Lys (termed FLAG), has been genetically fused to the C-terminus of the protease. Site-directed attachment to nonporous polystyrene and silica beads was achieved by employing the interaction between protein A and a monoclonal antibody specific for the FLAG peptide (Figure 4).

Cross-linked enzyme microcrystals (termed CLECs) are yet another interesting, although still rather expensive example of biocatalyst preparations which display high activities and stabilities, are readily handled, and can function efficiently in various media.[140] Recently, a comprehensive review was published by Margolin and Navia, the two pioneers of the CLECs- and CLPCs- (cross-linked protein crystals) technology.[141] In crystals, enzymes are ordered in a precise spatial arrangement originally joined together by valency bridges. As shown by X-ray diffraction analysis, the relatively large space be-

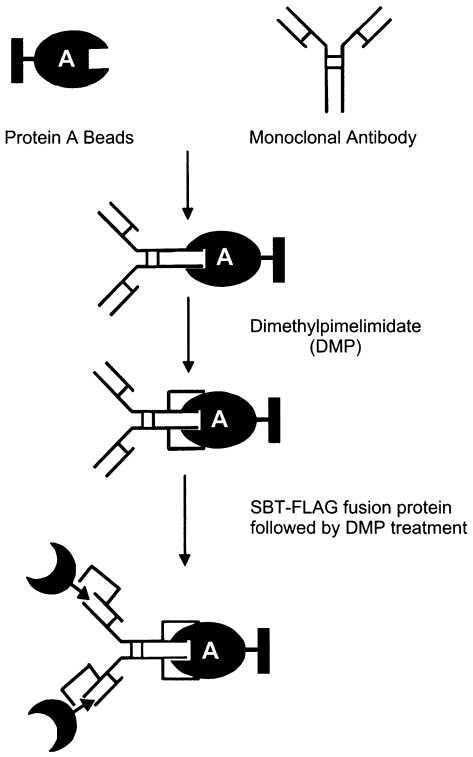


Figure 4. Schematic representation of the protein A-monoclonal antibody mediated site-directed immobilization of the subtilisin-FLAG fusion protein (according to ref 139 with permission from Springer-Verlag. Copyright 2001).

tween the enzymes usually contains water. Subsequent irreversible cross-linking inside the crystals with a bifunctional reagent such as glutaraldehyde locks the enzymes in the crystalline state outside of the conditions that led to crystallization. Generally, the cross-linking conditions must be carefully optimized to ensure both high stability and activity of CLEC catalysts. In this context, three major factors are important: (i) size of the crystal, (ii) size of the substrate, and (iii) conformation of the enzyme in the crystal. Additionally, mass transfer limitations on the substrates are known drawbacks of CLECs. Most of these problems can, in principle, be addressed by producing different crystal forms of the same enzyme. Several protease-CLECs have been successfully used for a number of regioselective acylations,[142] resolution of racemates,[143] the synthesis of peptides and peptidomimetics, and the mild hydrolysis of amino acid and peptide amides.[144] Subtilisin and thermolysin are by far the most often used protease-CLECs, and both of them are currently commercially available.

Dehydration of the enzyme, mostly performed by lyophilization, usually leads to conformational changes of the protein structure and, thus, decreases the enzyme activity when they are suspended in hydrophobic organic solvents (cf. Table 2). *Co-lyophilization with structure-preserving lyoprotectants*, such as substrate-resembling enzyme ligands,[145] sugars and polyols,[146] certain inorganic salts[147] or crown ethers,[148] often leads to enzyme preparations that are up to 4 orders of magnitude more active than those obtained in the absence of additives. Because of this high rate acceleration and the simplicity of the preparation by simple co-lyophilization this approach gained much

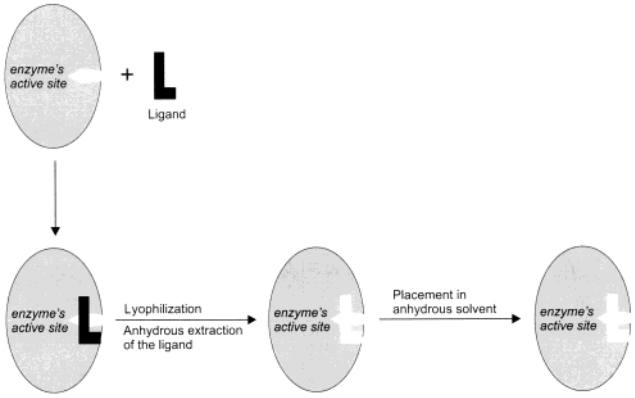


Figure 5. Schematic representation of the ligand-induced imprinting of the enzyme active-site (according to ref 50 with permission from Nature (http://www.nature.com). Copyright 2001).

attention. Various explanations exist to rationalize the basic individual reasons for this remarkable increase in enzyme activities. For the effect of substrate-resembling enzyme ligands on the enzyme activity a mechanism termed molecular imprinting is discussed.[145] As illustrated in Figure 5, the binding of those ligands to the active-site of the enzyme in water leads to a conformational change forming an imprint. This altered conformation of the enzyme's active-site remains after lyophilization, followed by extraction of the ligand with a suitable anhydrous solvent. Due to the structural rigidity of enzymes in organic solvents the ligand-induced highly active conformation remains unchanged in the anhydrous media. Besides improvements of the enzyme's activity, Rich and Dordick demonstrated that the nature of the ligand used for imprinting of subtilisin also allows for the rational control of enzyme specificities.[149] For example, dependent upon the nature of the imprint nucleoside, imprinting made possible the discrimination of subtilisin between structurally different, i.e., sucrose and thymidine, as well as structurally similar, i.e., thymidine and deoxyadenosin, nucleophiles. Parallel molecular modeling and molecular dynamic studies revealed that structural changes of the enzyme's active-site upon imprinting appear to be larger than originally thought influencing both the substrate binding and the catalytic machinery of the enzyme. The only apparent limitation to this approach is that the imprint ligand must be soluble in aqueous solution and must be soluble in the organic solvent reaction media for its removing.

The mechanism of enzyme activation by crown ethers is much more speculative than that of the active-site specific ligands. As for imprint ligands, there is a strong dependence of the degree of rate acceleration on the nature of the crown ether. Although all crown ethers used so far, i.e., 12-crown-4, 15-crown-5, and 18-crown-6, led to substantially enzyme activation, there are differences pointing to specific effects of crown ether activation in the individual reactions. Remarkably, rate accelerations up to 2 to 3 orders of magnitude are not unusual.[148] The acceleration, although varying, has been observed in different solvents (but not in water), for

different substrates, and for a number of proteases, such as subtilisin, chymotrypsin, and trypsin. Contrary to imprinting with ligands, crown ethers are effective without lyophilization, in principle, simply by adding them to the organic reaction mixture. Co-lyophilization, however, drastically increases the enzyme activity by orders of magnitude. Interestingly, extraction of the crown ethers after co-lyophilization with subtilisin by washing with THF did not "wash" the activating effect of crown ethers away, although it reduced the absolute enzyme activity 2-fold.[150] A similar result has been reported by van Unen and co-workers for chymotrypsin.[150] From these consistent findings, a molecular imprint-effect for crown ethers was postulated to be mainly responsible for the remarkable enzyme activation. Obviously, crown ethers act as likewise universal imprint ligands preventing structural changes of the active-site during the dehydration process. Contrary to imprint ligands, crown ethers dissociate from the enzyme's active-site when exposed to the organic solvents. With this explanation, however, it remains an open question why crown ethers are inactive toward acetyl-trypsin, in which acetylation of most of the trypsin's lysine side-chains is the only difference to the wild-type enzyme.[151] Nevertheless, the remarkable rate enhancement due to imprinting especially by crown ethers appears to be a general phenomenon and is certainly of high synthetic value which will probably gain much attention for a number of synthetic applications.

The smallest but one of the most effective lyoprotectants to date are inorganic salts such KCl. Originally, their use was prompted by the correlation between increased enzyme's activity and active-site polarity found within electron spin resonance spectroscopy studies on subtilisin used to investigate the role of essential enzyme bound water on the enzyme activity in organic solvents.[152] Thus, it is not surprising that it was a protease, i.e., subtilisin, for which the effect of enzyme activation by inorganic salts in neat organic solvents was first demonstrated. In fact, Khmelnitsky et al. found that the inclusion of excess KCl in an aqueous enzyme solution prior to freeze-drying afforded a dramatic 3750-fold enhancement in the catalytic efficiency of subtilisin in hexane over that of corresponding salt-free enzyme powders.[147] Further optimization of lyophilization time and water content even produced a catalyst with k_{cat}/K_m values of the same order of magnitude as that for the enzyme in aqueous media.[153] Studies from the same laboratory revealed that this activation was intrinsic and not due to reduced diffusional limitations.[154] It was further shown that this activation occurs only at extremely high levels of salt in the final enzyme preparation (98% w/w). Even a relatively small decrease from 98 to 90% w/w of salt significantly lowers the catalytic efficiency of the enzyme.[147,153] Salt-induced activation has also been observed for salts other than KCl, e.g., organic/inorganic buffers or neutral salts,[155] as well as other enzymes such as chymotrypsin,[147,155] thermolysin,[156] and lipases,[153,155] suggesting the generality of this phenomenon for a variety of enzymes in organic solvents. However, the exact mechanism of this salt-induced enzyme activation still remains a partly open question, although several studies are published in this context. Visualization of the freeze-dried enzyme preparations by scanning electron microscope technology revealed that the morphology of the catalyst changes from stringy, nodular structures to finer, particle-like formations as the salt concentrations increased from 0 to 98% w/w.[157] However, the alteration of the enzyme morphology occurs only at very high salt concentrations and not in a linear manner to the salt gradient. Griebenow and Klibanov investigated the effect of increasing salt concentration on the secondary structure of subtilisin by FTIR spectroscopy.[158] From the results, the researchers suggested that salt-activated subtilisin adopts a structure which is between that of the salt-free enzyme powder and that of subtilisin dissolved in aqueous solution. Obviously, the stabilization of the enzyme structure by the salt matrix during freeze-drying represents one reason for this remarkable salt effect. The improved stability of salt-activated enzymes in organic media probably by forming a protective salt shell around the enzyme,[146] the stabilization of the charged transition state by the increased active-site polarity afforded by the charged salt ions,[159] and preferential enzyme hydration by the salt matrix[160] are also expected to contribute to this effect significantly. Further improvements of the efficiency of salt-activated enzymes can be achieved by optimizing several key parameters such as enzyme preparation mode, nature of additives, or water content.[157,160] A more detailed picture covering the influence of additives on the activity of subtilisin is given in Figure 6. The most active subtilisin preparation were obtained by freeze-drying of the salt-activated enzyme at high cooling rates, by combining kosmotropic (water-structure making) and buffering salts having good freeze-drying properties at optimal molar concentrations (best results were obtained for 24.1% w/w $NaCH_3COO$ and 73.1% w/w

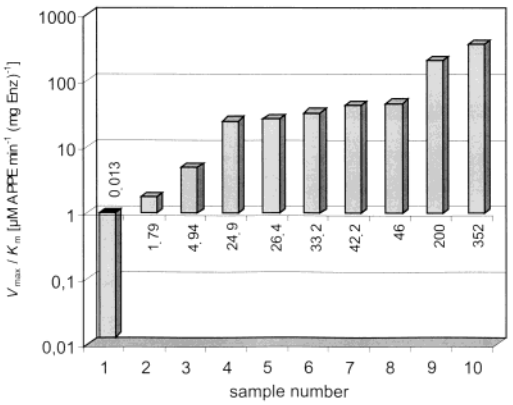


Figure 6. The progressive improvement of V_{max}/K_m for the transesterification of *N*-acetyl-phenylalanine ethyl ester (APPEE) in hexane by subtilisin activated by various combinations of additives (according to ref 157 with permission from John Wiley & Sons Inc. Copyright 2001). (1) salt-free enzyme, (2) PEG (MW = 2000), (3) 49% KCl/49% PEG, (4) KCl, (5) 49% $NaHCO_3$/49% PEG, (6) $NaHCO_3$, (7) 31.3% $NaHCO_3$/66.7% KCl, (8) $NaCH_3COO$, (9) 24.1% $NaCH_3COO$/73.9% $NaHCO_3$, (10) 24.1% $NaCH_3COO$/73.9% $NaHCO_3$ containing 0.8% water. In all cases, the total additive content was 98%.

$NaHCO_3$), and by adding small amounts of water (0.8% w/w). Presently, no further data are available from which the generality of these optimized parameters could be estimated. Similarly, only one single study exists that reports on the enantioselectivity of salt-activated proteases.[161] Accordingly, using KCl-activated subtilisin as the model catalyst, a variety of neat organic solvents as the reaction media, and the transesterification of *N*-acetyl-(L or D)-phenylalanine methyl ester and 1-propanol as the model reaction, only a marginal increase in enantioselectivity by about 30% over the salt-free enzyme has been observed which was nearly independent of the nature of organic solvent. This finding, although not inevitably representative, suggests that the activation by KCl is mostly due to a mechanism inherent to the enzyme rather than effects that favor one enantiomer over another. In conclusion, salt-activated proteases represent highly efficient and easily producible enzyme formulations for synthetic purposes while the large quantity of salts and, thus, the large quantity of the protease preparations required for a given level of product generation, appears to be an apparent drawback in practice.

The ionization state of enzymes is undoubtedly one of the most crucial parameters that control substrate binding, catalytic enzyme action, and three-dimensional structure of the enzyme molecule. In aqueous media, its importance is reflected by the pH profile of the enzyme's activity while the aforementioned "pH-memory effect" (cf. section 2) indicates a similar influence of the protonation state of enzymes on their catalytic activity in organic solvents. Although pH has no meaning in organic solvents, the protonation state of enzymes can be controlled by adding *buffering salts* consisting of an acid and its conjugated base. Presently, two different types of enzyme activating buffers are known: (i) soluble "organic phase buffers",[162] which represent a mixture of a Brønsted-Lowry acid and its conjugated base such as triisooctylamine/triisooctylamine hydrochloride, and (ii) so-called "solid-state buffers" such as Lys/Lys·HCl or Arg/Arg·HCl.[163] While the latter are insoluble in organic solvents, organic phase buffers are sufficiently hydrophobic compounds that even their ion pair salts are organic soluble in polar solvents such as 3-pentanone, dioxan, DMF, or THF. Mediated by the low dielectric of organic solvents the counterions of both buffer systems are closely associated with the enzyme molecule and, thus, control the protonation state of the enzyme in organic media independent of their solubility state. For cross-linked subtilisin crystals Xu and Klibanov have been observed that the degree of maximal activation by organic phase buffers strongly correlated with the pK_a values of the acid.[164] Stronger acids and hence weaker conjugated bases generally afforded far lower activation effects (less than 1 order of magnitude) than weaker acids (and hence stronger bases), up to 2 orders of magnitude. Maximal activation was reached when phenylboronate/sodium phenylboronate was used as the organic phase buffer system whereas a ratio of acid and base of 1:4 has been found to be optimal. A similar correlation of the acid's pK_a value and the enzyme activity was found for solid-state buffers.[165] On the contrary, neither the total amount nor the ratio of solid acid and base affected the degree of enzyme activation. A further advantage of solid-state over organic phase buffers results from their nonsensitivity toward alterations of the organic solvent.[163] Hence, an acid/base-pair found optimal for a particular enzyme should be valid for all hydrophobic organic media in which ion-pairing remains complete. Interestingly, the expectation that ion exchange between the solid-state buffers and the enzyme molecule is a rather slow process was found to be wrong. Both direct approaches that monitored changes in the acid−base conditions in situ[165] and indirect measurements using the synthesis activity of subtilisin as an indicator[163] gave no hints to any serious limitations caused by the rate of ion exchange. In closing, the use of buffering salts, in particular of solid-sate buffers, offers an inexpensive and rather general way of precisely controlling the protonation state of an enzyme in organic solvents. It makes lyophilization or immobilization at a whole series of different pH values to find the optimum no longer necessary. Moreover, changes in the ionization state of the enzyme during reaction caused by the formation of charged synthesis products which usually lead to a loss of enzyme activity can be completely avoided.

Despite the large number of papers that report on techniques to the stabilization of proteases originally less stable in organic solvents, only a very few attempts have been made to screen for *natural enzymes with improved organic solvent tolerance*. Ogino and Ishikawa recently summarized the current state of research in this field in a short review.[166] According to this, less than 10 actual papers exist on this topic reporting on only two organic solvent stable proteases, i.e., *Thermus* strain Rt4A2 protease[167] and *Pseudomonas aeruginosa* PST-01 protease,[168] while most of the work stems from the authors themselves. Up to now, only the latter enzyme was used for synthetic applications indicating not only a high organic solvent stability of the enzyme but also remarkable reaction rates and product yields.[169] Addressing the molecular reasons for this improved organic solvent tolerance is certainly an issue of general importance, but was subjected by only two initial papers.[170] On the basis of sequential and structural alignments, PST-01 protease was found to be highly homologous to thermolysin with the exception that PST-01 protease contains two disulfide bonds. Despite this homology, the two enzymes exhibit considerably different stabilities in organic solvents (Table 3). Site-directed mutagenesis studies gave hints to an important role of at least one of these disulfide bonds in the improved organic solvent stability of PST-01, but cannot explain the distinct solvent tolerances of the two proteases completely. Interestingly, the improved stability of PST-01 in organic solvents was not accompanied by an improved thermostability which is sometimes considered to be closely related.

4. Synthesis in Nonconventional Media

Neat organic solvents or aqueous−organic mixtures, although most popular, are not the only

Table 3. Half-Life of *Pseudomonas aeruginosa* PST-01 Protease and Thermolysin in Organic Media[a]

solvent	half-life (day)	
	PST-01 protease	thermolysin
ethylene glycol	>100	>50
1,4-butandiol	>100	4.4
1,5-pentandiol	>100	1.7
ethanol	>100	3.0
1-hexanol	>50	18.2
methanol	>50	4.6
DMSO	>50	2.6
2-propanol	>50	1.2
triethylene glycol	>50	5.1
tert-butanol	>50	0.8
1-heptanol	>50	13.1
DMF	25.3	0.9
1-octanol	24.2	n.t.
1-butanol	24.2	0.9
acetone	23.1	0.7
1-decanol	19.4	n.t.
1,4-dioxan	17.7	0.8
toluene	12.0	22.5
benzene	7.8	n.t.
n-heptane	4.8	n.t.
p-xylene	4.4	n.t.
n-hexane	3.8	n.t.
n-decane	2.4	n.t.
cyclohexane	2.3	n.t.
aqueous medium	9.7	10.8

[a] According to ref 168b with permission from the Society of Biosciences and Bioengineering. Copyright 1999. To solutions of 3 mL containing the appropriate protease 1 mL of organic solvent was added and the residual activities were measured for 15 days. The half-lives reported were calculated from the exponential regression curves. n.t., not tested.

approaches useful for medium engineering related to changes in enzyme properties. A number of other interesting techniques contribute to this field including supercritical fluids, frozen aqueous media, supercooled organic solutions, or solid-to-solid conversions. The term "nonconventional" for this media has been chosen because their application for synthesis still remains an exception rather than the routine. Some of them are closely related to organic solvents such as supercritical fluids and supercooled organic mixtures, while others, e.g., frozen-state syntheses, usually uses frozen water as the solvent. Solid-to-solid conversions are described for both media although their amount is marginal in such systems.

The first use of *supercritical fluids* as reaction media for enzymes dates back to 1985.[171] Since those first experiments several hundred papers were published. Recently, this list was continued by a comprehensive review published by the laboratory of Russell who is presently one of the most active scientists in this field.[172] The enzymes employed in most of the work involving supercritical fluids have been lipases. But even for these enzymes, the papers published so far mainly reports on basic mechanistic studies and small-scale model syntheses which may reflect the current state of this technology for biocatalysis. Supercritical fluids are materials above their critical temperature and critical pressure and can be considered in a more general term as compressed gases. The properties of those fluids lie between that of liquids and gases. For example, supercritical fluid densities are comparable to those of liquids, while the diffusivities and viscosities are closer to those of gases. Hence, especially reactions that are limited by mass transfer rates profit from the gaslike diffusivities and low viscosities and, thus, usually proceed faster in supercritical fluids than in classical organic solvents.[173] It has been stated that the main advantage of this technology is that the physical properties of supercritical fluids have the ability to be manipulated by merely changing the temperature or pressure of the reaction system.[174] For example, small changes in pressure lead to significant changes in density, which in turn alters all density-dependent solvent properties, such as dielectric constant, solubility parameter, and partition coefficient.[175] Since the resultant changes in properties are predictable, one can rationally control all aspects of the reaction environment and, thus, the biocatalytic reactions themselves. Kamat et al. for example highlighted this behavior for subtilisin- and *Aspergillus* protease-catalyzed transesterification reactions of *N*-acetyl-(L or D)-phenylalanine ethyl ester with methanol.[176] Both proteases became more stereoselective as the pressure and, hence, the dielectric of the supercritical fluid was increased. Other studies were conducted by Chaudhary et al. that further demonstrated that not only the selectivity and specificity but also the activity of subtilisin changed as the pressure was altered.[177] Similarly, the enzyme stability was found to be at least partly a function of the pressure of the reaction system.[178] Additionally, the nature of the supercritical fluid itself influences the catalytic behavior of the enzyme. Although carbon dioxide is by far the most popular solvent due to its low toxicity and cost, it has a negative effect on the stability and activity of most enzymes.[179] The formation of covalent carbamate−enzyme complexes,[180] the alteration of the protonation state of the enzyme by carbon dioxide,[181] and changes of the enzyme conformation[182] are discussed to be mainly responsible for this behavior. Compressed ethane, propane, or fluoroform exhibit better characteristics and are now recognized to be a more suitable choice for biocatalytic reactions.[183] Although the practical utility of supercritical fluid systems is beyond doubt and offers intriguing opportunities for tailor-made high-performance applications, the advantages of replacing conventional organic solvents with supercritical fluids have not fully been demonstrated yet. One reason may be the size of equipment and the high capital and operating costs of this technology in particular for continuous synthesis processes.

Freezing of aqueous or organic solutions does not inevitably mean the dead end of an enzymatic reaction in every instance as it has been widely expected empirically or, in more rational way, by extrapolation of the Arrhenius plot. This misconception has been already refuted in 1939 by initial investigations of Lineweaver[184] and Sizer and Josephson[185] who could show that proteases remain active under frozen-state conditions. Newer systematic studies on the effect of freezing on enzymatic reactions have established "cryo-bioorganic chemistry" as its own field of research with intriguing opportunities for organic synthesis. To separate it from others, cryo-bioorganic

Table 4. Influence of Freezing on the Coupling of Specific Amino Acid-Containing Peptides Catalyzed by Trypsin, *Bacillus Licheniformis* Glu-Specific Endopeptidase (BL-GSE), and Chymotrypsin[a]

		yield (%)	
protease/acyl donor	acyl acceptor	25 °C	−15 °C
	Trypsin		
Bz-Arg-OEt	H-Ala−Ser-OH	12.0	93.5
	H-Ala−Ala-Lys-Ala-Gly-OH	39.5 (51.3)	82.9
	H-Ala−Ala-Arg−Ala−Gly-OH	39.9 (52.6)	89.9
	Chymotrypsin		
Ac-Tyr-OEt	H-Gly−Leu-NH_2	69.6	97.5
	H-Ala−Ala−Phe−Ala−Gly-OH	59.9 (68.8)	89.1
	H-Ala−Ala−Tyr−Ala−Gly-OH	61.2 (71.3)	90.1
	BL-GSE		
Z-Glu-OMe	H-Gly−Leu-NH_2	7.5	94.0
	H-Ala−Ala-Asp−Ala−Gly-OH	8.0	94.5
	H-Ala−Ala−Glu−Ala−Gly-OH	11.4	94.7

[a] According to ref 191. The specific amino acid moieties within the nucleophilic peptides are underlined. Brackets generally indicate competitive peptide cleavages. The data in brackets correspond to the whole peptide products while the data outside give the yields of the appropriate intact hexapeptide product. Conditions at 25 °C: 0.1 M Hepes buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-8} M, [chymotrypsin] = 1.0×10^{-8} M, [BL-GSE] = 1.0×10^{-8} M. Conditions at −15 °C: distilled water (pH 8.0 before freezing), [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-6} M, [chymotrypsin] = 1.0×10^{-7} M, [BL-GSE] = 1.0×10^{-8} M.

chemistry is focused on a temperature range wherein no eutectics are formed. Contrary to eutectic systems, in which all components of the reaction mixture are in their solid-sate, frozen-state reactions can be considered as two-phase systems consisting of the frozen crystalline solvent and a diminished liquid phase of cavities. The latter contains the enzyme and the reactants in a highly concentrated fashion, a situation that is known as "freeze-concentration effect".[186] In other words, freezing is actually the equivalent of partial drying, because it means the removal of water in form of ice from the solution, and by this manner the freeze concentration can result in supersaturated solutions of the reactants in which the solutes can be concentrated even in 0.1% of the original liquid volume.[187] This unique composition of the reaction mixture mediates a unique enzyme behavior that was subjected by a number of papers. A few recent reviews contribute to this list summarizing actual efforts in cryo-bioorganic chemistry in a more or less comprehensive manner.[188] Accordingly, kinetically controlled protease-catalyzed peptide synthesis is currently the major field of application of this approach and was pioneered by the laboratory of Jakubke.[189] It could be established that amino acid derivatives, peptides, and even free amino acids which are considered to be inefficient nucleophilic amino components at room temperature provide significantly higher yields under frozen-state conditions at temperatures between −10 and −25 °C. Furthermore, N^{α}-unprotected dipeptide esters and acyl donors containing noncoded building blocks could be successfully coupled in frozen reaction mixtures.[190] This high synthesis efficiency is accompanied by a reduced competitive hydrolysis activity of the enzyme. This equally holds true for the acyl donor ester, the newly formed peptide bond, and cleavage sensitive amino acid moieties within the peptide reactants as it has been recently shown for trypsin-, chymotrypsin-, and *Bacillus licheniformis* Glu-specific endopeptidase-catalyzed reactions (Table 4).[191] Mechanistic studies on protease-catalyzed peptide synthesis under frozen-state conditions revealed that there are probably other factors than the freeze-concentration effect involved in peptide yield enhancement. Using the ^{1}H NMR relaxation time technique, the amount of unfrozen water has been determined in frozen samples at −15 °C and compared to relaxation time measurements for the same system at room temperature.[192] An apparent concentration factor of 50 for the unfrozen water concentration was determined. However, simulation of these concentration relations at room temperature in papain-catalyzed peptide synthesis experiments could not confirm the yield-increasing effect. Therefore, freeze-concentration was concluded to be not the only cause of the yield-increasing effect. Other physical and reaction parameters must be considered, e.g., increased proton mobility in ice, changes of dielectric behavior, reduced water activity, and imposition of a favorable orientation of substrate and enzyme. It should be noted, however, that Tougu et al. studied the same effect in a similar manner in frozen and liquid solutions at −18 and +20 °C, and they concluded that the concentrated solutions match the conditions of the unfrozen liquid phase in ice.[193] Thus, further investigations are needed to clarify this contradiction. Whatever the real mechanism, however, protease-catalyzed peptide synthesis in frozen systems exhibits a large number of advantages including: (i) significant yield enhancement; (ii) suppression of competitive hydrolytic side reactions; (iii) coupling of originally inefficient amino components; (iv) high stability of chemically labile reactants; and (v) abandonment of the protection of additional reactant functionalities. Because of these benefits, freezing the aqueous media could be an excellent technology to avoid the use of organic solvents which are problematic especially for application in the food sector. As already outlined for supercritical fluids, the size of equipment and the high capital and operating costs connected with the freezing process may by considered as general drawbacks of the frozen-state technology in particular for continuous large-scale syntheses. Finally, it should be noted that a couple of papers report on an

unsuccessful use of frozen solutions in dipeptide synthesis indicating that the effects of freezing may not be generalized.[194]

A freezing-like effect on the product yields and competitive hydrolytic side reactions, although significantly less pronounced, is reported for *supercooled organic solvent systems*.[195] It was found that this approach works for a large number of proteases as well as various organic media differing in the nature of the organic solvent or their water content. The individual effects on both parameters, however, can vary and are not predictable presently. While Nilsson et al.[196] reported for the synthesis of Ac-Tyr−Ala-NH_2 by chymotrypsin an increase in the reaction yield from 8.7% at room temperature to about 97% at −35 °C, similar studies of Jönsson et al.,[197] which only differed in the nature of the acyl donor (Ac-Phe-OEt instead of Ac-Tyr-OEt) and the reaction temperature (−1 °C instead of −35 °C), revealed yield enhancements of 20% on average. Nevertheless, in almost all instances improved synthesis yields and product stabilities could be observed at subzero temperatures than under normal conditions. The authors suggest a different dependency of the aminolysis versus hydrolysis reactions, i.e., lower temperature decreases the water activity to a larger extent than it influences the reactant activities, dissolved in the organic media. Thus, in cases where low yields and undesired hydrolysis hinder the enzymatic reaction, decreasing the temperature to subzero ranges represents an easy approach that often leads to significant improvements of the efficiency of synthesis.

Complete dissolution of reactants and enzymes in the reaction mixture is usually considered to be essential for efficient synthesis and, as noted above, a lot of efforts have been done to improve the solubility of the reactants or to increase the solubilizing power of the solvent. In this context, it may be highly surprising that approaches have been recently developed that report on "*solid-to-solid conversions*",[198] "*heterogeneous eutectic mixtures*",[199] or "*solid-phase syntheses*".[200] Although applying distinct terminologies, all three approaches are essentially based on the same class of reaction system, i.e., synthesis with mainly undissolved reactants at very high concentrations. Halling and co-workers recently summarized the characteristics of such reactions and have discussed their basic kinetics and thermodynamics as far as they are already know.[201] Accordingly, the term "solid-to-solid" conversion was proposed for further general use, although it was pointed out that none of the designations introduced so far meet this sort of reaction system perfectly. For example, the term "solid-to-solid" may give the mistaken impression that the enzymatic reaction occurs in a solid-state which is, however, not the case. In fact, the reaction requires an essential liquid phase as it has been proven by infrared microscopic studies[202] as well as experiments analyzing the yields as a function of the amount of added water.[203] The volume of essential liquid phase, however, can be very small. It was found that the amount of water adhered to salt hydrates[198] or on commercially available substrate and enzyme preparations as the only water source, which was determined to be 0.5% (w/w) at its lowest, was sufficient to mediate enzymatic syntheses.[204] However, more often the liquid phase is created by adding traces of a third component, i.e., water or an organic solvent, which is liquid at the reaction temperature. In general, 10% (w/w) "liquid phase" is typically used in such systems while both an increase and a decrease of the solvent volume lower the efficiency of synthesis.[204] The influence of organic solvents as the liquid phase on the enzymatic reaction was summarized by Lopez-Fandino et al.[205] The authors found that the nature of the organic solvent affects primarily the composition and physicochemical properties of the liquid phase which secondarily influences the enzyme activity, product crystallization, and finally the yield of the reaction. In general, hydrophilic oxygenated solvents proved to be very effective. In addition, and contrary to common belief, a more efficient conversion has been achieved in media in which the reactant solubility is poor, rather than in highly solubilizing solvents. A similar effect was recently reported for cosolvents which were added to improve reactant solubilities.[206] The successful application of this technology for a wide spectrum of protease-catalyzed syntheses may be misleading that only little is known about the thermodynamics of such systems. First, theoretical attempts published by Halling et al. led to a model that describes a linear dependence of the equilibrium yield on the reciprocal of the initial reactant concentrations and has been validated experimentally in the same paper.[207] However, further efforts are needed to explain why the reaction displays a switch-like behavior under solid-to-solid conditions which directs the reaction to product formation until all insoluble reactants are consumed. From a synthetic point of view, the use of the solid-to-solid approach brings valuable benefits. It combines the equimolar or nearly equimolar supply of reactants with high obtainable yields and high ratios of product to reaction mixture volume, easy workup procedures, and, in principle, compatibility with conventional chemical peptide synthesis standard procedures. In addition, it avoids the use of huge amounts of organic solvents and, hence, may have an additional interest in designing processes with improved safety and environmental friendliness. Several preparative scale syntheses have been already reported (cf. chapter IV), while mainly technical problems such as mixing of the reactants and the enzyme were found to be the most serious problems in the scale-up procedure.[203,208] Additional shaking and/or ultrasonication was found to be necessary to prevent sticking of the enzyme and reactants to the wall of the reactor. Further research should address this technical problem obviously connected with large-scale synthesis and should be focused on the synthesis of interesting targets which would certainly further improve the attraction of this powerful technology.

B. Substrate Engineering

If undesired subsequent reactions are observed during kinetically controlled synthesis reactions, then it is of minor importance which bond is cleaved by

the enzyme. These side reactions merely show that the specificity of the enzyme for the acyl donor ester does not lie sufficiently above its specificity for the peptide product. Since structural changes in the amino acid building blocks of the reactants are ruled out, the leaving group of the acyl donor ester remains as the only variable to suppress competitive reactions. To circumvent this limitation, specific leaving group manipulations at the carboxyl component are commonly used in the kinetic approach. Efforts in this direction enabled syntheses using originally protease-labile peptide reactants and finally led to the development of the so-called "substrate mimetics" approach which will be a major subject in the following section.

1. Classical Concept of Leaving Group Manipulation

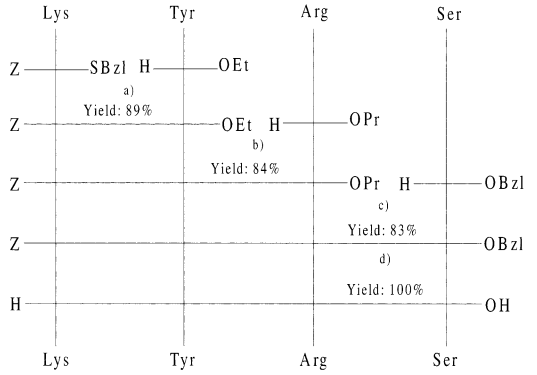
The structural requirement for a protease substrate is the presence of specific amino acids in the acyl moiety, for example, Arg or Lys in the case of trypsin. Hence, acyl donor components lacking those site-specific amino acid residues at their C-terminus are usually no targets for protease-catalyzed syntheses. Although most proteases recognize more than one amino acid moiety, the spectrum of the currently known enzymes does not enable all proteinogenic sequences to be coupled. Compounds containing noncoded amino acids at the C-terminus of the acyl donor ester are generally not substrates under normal conditions. Moreover, due to the distinct specificity of proteases toward the amino acid moieties which are recognized by the enzyme, only acyl donors possessing the most specific amino acid residue at the C-terminus can be coupled without serious proteolytic side reactions. In contrast, the coupling of the less specific amino acid moieties only proceed successfully if the peptide reactants do not contain any of the more specific counterparts. For example, the endoproteinases Glu-C from *Staphylococcus aureus* strain V8 (V8 protease) or from *Bacillus licheniformis* (BL-GSE) specifically recognize glutamic acid and aspartic acid as well. However, the specificity of the enzymes toward both residues differs by about 3 orders of magnitude, while V8 protease and BL-GSE are more specific for glutamic acid.[209] Hence, coupling of Asp−Xaa bonds only proceeds successfully with the two enzymes if there is no glutamic acid in the sequence of the reactants. Reversely, Glu−Xaa bonds can be synthesized without taking care on the presence of additional aspartic acid moieties. In instances in which the differences in the specificities are less pronounced, leaving group manipulation can be used to improve the specificity of the originally less specific acyl donor over that of the more specific and, thus, cleavage sensitive amino acid moiety located within the reactants. The general function of this approach could be already demonstrated 10 years ago by the laboratory of Jakubke.[210] Several recent papers continue to this work including the use of more than 30 different types of esters especially for chymotrypsin-mediated syntheses.[211] In addition, leaving group manipulations were found to be generally useful to increase the enzyme's activity toward originally less specific acyl donors and, thus, to accelerate the rate of reaction. Typical rate and specificity improvements that can be achieved toward such poor substrates are illustrated in Table 5. In particular, preparative scale syntheses profit from the higher reaction rates of leaving group engineered acyl donors. For example, about 90% of the expensive protease clostripain could be saved simply by using Z-Lys-SBzl instead of Z-Lys-OMe as the acyl donor component in the first reaction step of the synthesis of the highly trifunctional tetrapeptide H-Lys−Tyr−Arg−Ser-OH (Scheme 4).[212] Contrary to the effect on the reaction rate, only a marginal influence of the nature of the leaving group on the ratio of hydrolysis and aminolysis of the acyl donor and, thus, on the product yields are expected. This is because of the formation of identical acyl enzyme intermediates which should undergo an identical partitioning into the peptide product and the competitive hydrolyzed acyl donor, respectively. Leaving group related differences in product yields may only result from incomplete acyl donor consumption or differences in the rate of spontaneous hydrolysis of the distinct acyl donor esters.

Table 5. Influence of the Ester Leaving Group on the Specificity of the Acyl Donor toward Trypsin and Clostripain

protease/substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Trypsin[a]			
Mal-Phe-OCH_3			1.00×10^2
Mal-Phe-OCH_2CF_3	3.82	82.0	2.15×10^4
Mal-Phe-$OCH(CH_3)CONH_2$	3.79	122.8	3.24×10^4
Mal-Phe-SCH_2CONH_2	3.76	193.6	5.15×10^4
Mal-Phe-$OCH_2CONHBzl$	13.1	74.9	5.72×10^3
Mal-Phe-$OCH_2CONHPh$	13.4	94.3	7.04×10^3
Mal-Phe-OCH_2CO−Phe-NH_2	8.82	160.5	1.82×10^4
Clostripain[b]			
Bz-Lys-OCH_3	7.24	187.1	2.58×10^4
Z-Lys-OBzl	2.07	109.0	5.27×10^4
Z-Lys-SBzl	0.34	118.2	3.48×10^5
Z-Lys-ONp	0.22	98.0	4.45×10^5

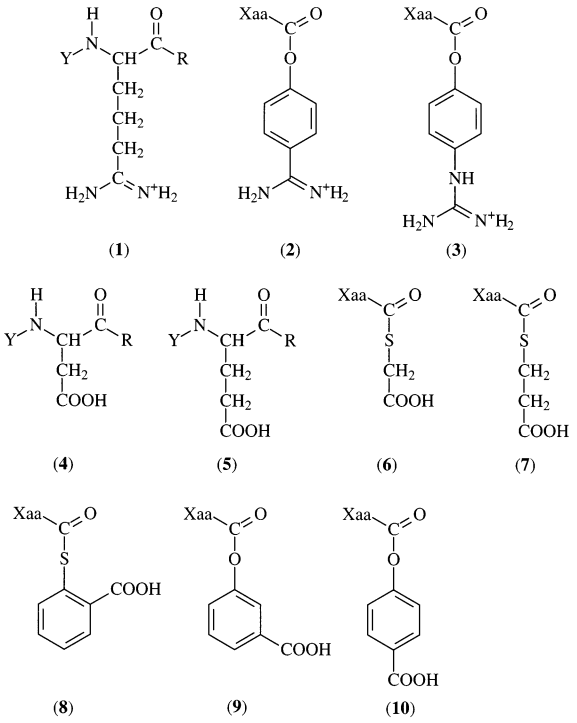
[a] Elsner, C. Ph.D. Thesis, University of Leipzig, 2000. [b] Bordusa, F. Ph.D. Thesis, University of Leipzig, 1998.

Scheme 4. Scheme of the Protease-Catalyzed Synthesis of the Tetrapeptide H-Lys−Tyr−Arg−Ser-OH in Preparative Scale Using the Leaving Group Optimized Z-Lys-SBzl as the Starting Acyl Donor of the Stepwise Synthesis[a]



[a] According to ref 212. (A), (C) clostripain, 0.025 M borate buffer, pH 8.0, 25 °C; (B) chymotrypsin, 0.025 mM borate buffer, pH 9.0, −25 °C; (D) catalytic hydrogenation using 10% Pd.

Scheme 5. Structures of Substrates and Substrate Mimetic Moieties for Arg- and Glu-Specific Proteases[a]



[a] (1) Arginine; (2) 4-amidinophenyl ester; (3) 4-guanidinophenyl ester; (4) aspartic acid; (5) glutamic acid; (6) carboxymethyl thioester (SCm); (7) carboxyethyl thioester (SCe); (8) 2-carboxyphenyl thioester (S2Cph); (9) 3-carboxyphenyl ester (O3Cph); (10) 4-carboxyphenyl ester (S4Cph).

2. Substrate-Mimetics Mediated Syntheses

Contrary to classical leaving group manipulations that are focused on the adaptation of the leaving group structure to meet the specificity of the S′ subsite of proteases, in substrate mimetics the leaving group is fitted to bind into the active-site (S_1 position) of the enzyme. Nature itself can be considered as the prototype of this kind of substrate engineering. The ribosomal peptidyl transferase, which is the nature's expert for peptide bond formation, indeed does not recognize the amino acid itself that is subjected for coupling, but the tRNA of which it is esterified. This strategy enables peptide synthesis in both a specific and universal manner thereby applying only a single enzyme to catalyze the peptide bond formation step. In substrate mimetics, the shift in the location of the site-specific moiety from the peptide's C-terminus into the leaving group is equally accompanied by a shift in the enzyme activity, enabling serine and cysteine proteases to react with nonspecific amino acid or peptide sequences. Importantly, for this remarkable activity no further manipulations either on the enzyme nor the reaction medium are, in principle, necessary. The first examples of such substrate mimetics reported were acyl-4-amidino and -4-guanidinophenyl esters (OGp) (Scheme 5, compounds **2** and **3**) which were found to be recognized by originally Arg-specific proteases nearly independent of their individual peptide sequence.[213] Interestingly, this nonspecificity for the acyl moiety not only holds for coded L-amino acids,

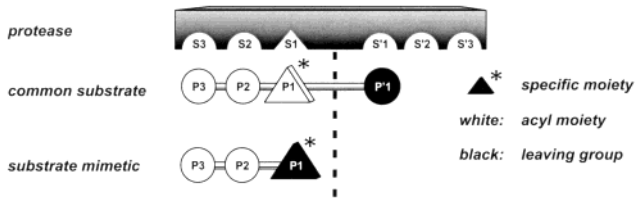


Figure 7. Schematic comparison of the binding of substrate mimetics and common acyl donor components to the active-site of trypsin based on the ideas of the conventional binding model of proteases. Due to the binding of the site-specific ester leaving group in place of the specific amino acid moiety of common substrates, the scissile ester bond of substrate mimetics would be far away from the active amino acid residue of the protease and, thus, formation of the acyl enzyme intermediate cannot occur.

but also for their D-configured counterparts and even for α,α-dialkylated amino acids. Although this type of esters has been first reported in 1973 within studies on the inhibition of Arg-specific proteases,[214] only very little was known about the basic mechanism of binding and hydrolysis of substrate mimetics. Applying the familiar model of substrate recognition that holds for classical protease substrates leads to a catalytically unproductive orientation of the substrate mimetics within the active-site of proteases as illustrated by Figure 7. To demystify the mechanism of catalysis, we recently used experimental and theoretical approaches in a unique combination utilizing trypsin as the model catalyst and acyl-4-guanidinophenyl esters as the model substrate mimetics.[213g] We found that the specific OGp group binds indeed at the specificity determining S_1 position of the protease adopting a similar arrangement as it is found for specific Arg-residues of common acyl donors (Figure 8a,b). Interestingly, despite the OGp binding at the enzyme's S_1 position the scissile ester bond of substrate mimetics is exactly located at the same position as that of classical protease substrates. On analysis of the orientation of both types of substrates, this arrangement results from a reverse binding of the substrate mimetics that do not bind at the S subsite of the enzyme as it is known for normal substrates, but at the enzyme's S′ subsite. Further experimental studies including investigations on the dependence of the hydrolysis as well as the reverse of hydrolysis on the individual acyl residue of the substrate mimetics already supported this unique catalysis mechanism.[213g]

Recent reports on the substrate mimetics-mediated approach has been illustrated its synthetic utility for the ligation of peptide fragments in addition to single amino acid containing acyl donor and acceptor components used so far.[213d] Furthermore, studies with other Arg-specific proteases than trypsin, such as thrombin or the cysteine protease clostripain, have been indicated that the concept of substrate mimetics is neither restricted to trypsin nor to serine proteases.[213d,215] A further important advantage of substrate mimetics-mediated syntheses directly results from the coupling of nonspecific sequences. Consequently, in contrast to enzymatic reactions using normal-type acyl donors, the peptide bond formed cannot be recognized by the biocatalyst and, therefore, is not subject to secondary cleavage. As shown

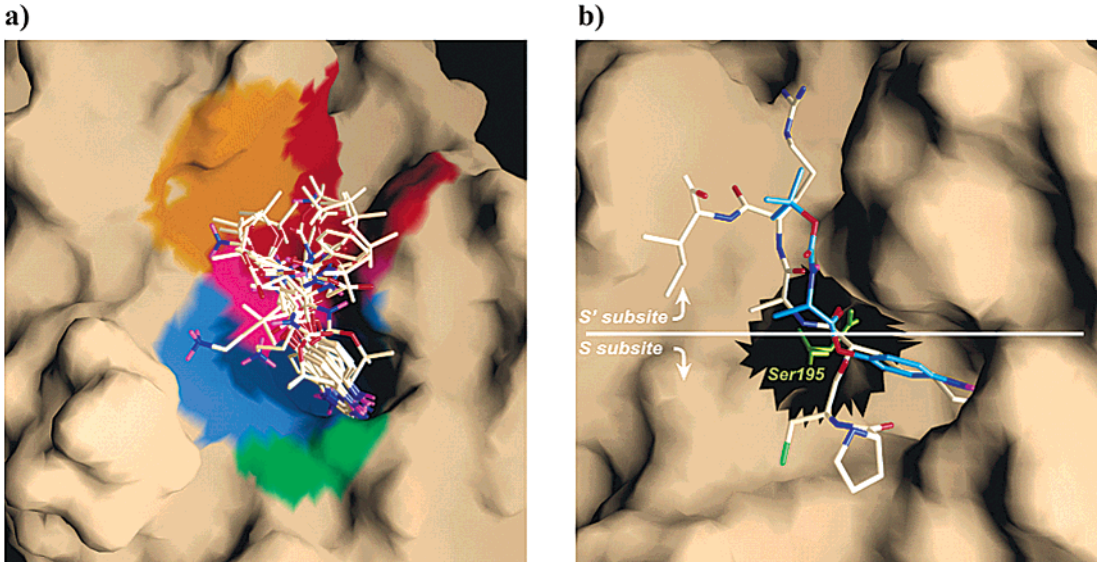


Figure 8. Binding of Boc-Xaa-OGp to trypsin (according to ref 213g). (a) Calculated lowest-energy productive complexes of Boc-Xaa-OGp and trypsin. Xaa: Gly, L-Ala, D-Ala, L-Leu, D-Leu, L-Gln, D-Gln, L-Glu, D-Glu, L-Phe, D-Phe, L-Lys, and D-Lys. The regions of the subsites S_3 (green), S_2 (light blue), S_1 (blue), S'_1 (pink), S'_2 (red), and S'_3 (orange) are also shown. (b) Comparison of the binding conformation of the $P_3-P'_3$ residues of BPTI (Pro13−Cys14−Lys15−Ala16−Arg17−Ile18), that binds in the mode of a common substrate, and the lowest-energy state of Boc-L-Ala-OGp to the active-site of trypsin. BPTI (white), Boc-L-Ala-OGp (blue).

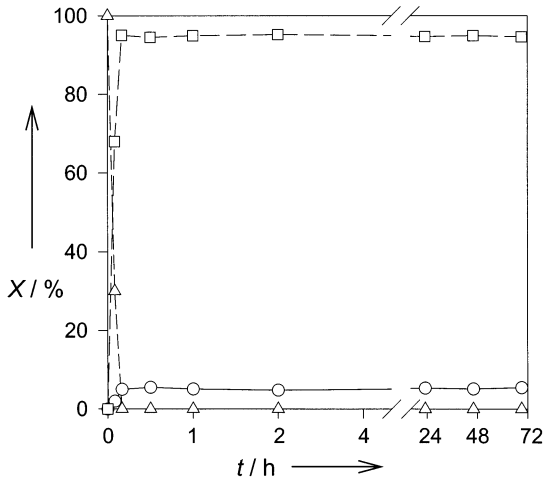


Figure 9. Course of the clostripain-catalyzed (3+5) segment condensation of Boc-Phe−Gly−Gly-OGp and H-Ala−Phe−Ala−Ala−Gly-OH (according to ref 213d). Boc-Phe−Gly−Gly-OGp (△); Boc-Phe−Gly−Gly−Ala−Phe−Ala−Ala−Gly-OH (□); Boc-Phe−Gly−Gly-OH (○). Conditions: 50 mM HEPES-buffer, pH 8.0, 100 mM NaCl, 10 mM $CaCl_2$, 25 °C; [clostripain] = 1.6×10^{-6} M; [acyl donor] = 2 mM; [H-Ala−Phe−Ala−Ala−Gly-OH] = 4 mM; X = product yield.

by the course of the (3+5) segment condensation (Figure 9) the maximum concentration of the product Boc-Phe−Gly−Gly−Ala−Phe−Ala−Ala−Gly-OH achieved after a few minutes remains unchanged after 72 h.[213d]

Although the binding and kinetics of substrate mimetics follow a unique mechanism, there are no hints to a general restriction of the substrate mimetics approach to trypsin or trypsin-like proteases. Recent studies on the Glu-specific V8 protease already supported this assumption establishing the first substrate mimetics, i.e., carboxymethyl thioesters (SCm), for nonarginine-specific proteases.[216] The carboxymethyl thioester functionality (**6**) was selected empirically based on the close structural similarity to the side-chain of specific Asp (**4**) and Glu (**5**) residues (cf. Scheme 5). Interestingly, despite the highly restricted specificity of V8 protease, the carboxymethyl thioester group was found to act as a suitable mimic mediating the acceptance of originally nonspecific acyl moieties as it was found for the OGp esters and trypsin-like proteases. Recent efforts to design more specific mimetic moieties achieving higher reaction rates with lower enzyme necessities led to number of further types of substrate mimetics for Glu-specific enzymes (compounds **7**−**10** Scheme 5).[217] It has been found that aromatic rather than aliphatic linkers between the binding site specific moiety and the nonspecific acyl residue lead to an increase in specificity of substrate mimetics and, hence, to an increase in the reaction rates. On the contrary, model peptide syntheses have been shown that the individual leaving group only affects the yield of the reactions to a very small extent as it has been already discussed for classical leaving group optimized acyl donors (cf. foregoing section).[217]

Recently, the extension of the substrate mimetics approach to a third synthetically important class of proteases, i.e., enzymes that are specific for aromatic and uncharged amino acid moieties, has been described on the example of chymotrypsin.[218] A computer-assisted protein−ligand docking approach has been used in this study to predict the function of the 4-guanidinophenyl ester functionality to act as an artificial recognition site for this enzyme utilizing Boc-L-Ala-OGp as a model ligand. The OGp group itself was selected due to its bifunctionality combining both a recognition site for Arg-specific enzymes (guanidino part) and for proteases bearing a specificity for aromatic amino acids (phenyl part). Figure 10 shows the calculated arrangements of the ligand in the active-site of chymotrypsin in the lowest-energy complex in comparison to that formerly found with

Table 6. Yields (%) of Chymotrypsin-Catalyzed Peptide Syntheses Using 4-Guanidino-Phenyl Esters Bearing Noncoded Amino Acid and Carboxyl Acid Derivatives[a]

	acyl donor					
acyl acceptor	Bz-L-Ala-OGp	Bz-L-Glu-OGp	Bz-L-Pro-OGp	Bz-D-Ala-OGp	Bz-D-Leu-OGp	Bz-D-Phe-OGp
H-Arg-NH_2	79.9	91.6	98.5	94.9	92.2	78.5
H-Met-NH_2	62.3	77.9	97.7	93.0	75.0	66.8
H-Leu-NH_2	50.4	56.7	88.5	88.9	67.5	50.2
H-Ala−Ala-NH_2	75.9	66.9	96.3	95.7	77.6	55.0
H-Gly−Leu-NH_2	80.9	54.6	94.0	95.3	90.5	52.0
H-Leu−Ala-NH_2	72.5	58.1	99.0	79.7	79.6	56.5

[a] According to ref 218. Conditions: 0.2 M HEPES-buffer, pH 8.0, 0.2 M NaCl, 0.02 M $CaCl_2$, 25 °C, 10% MeOH; [acyl donor] = 2 mM, [acyl acceptor] = 20 mM, [chymotrypsin] = 37 μM; reaction time: 5 min.

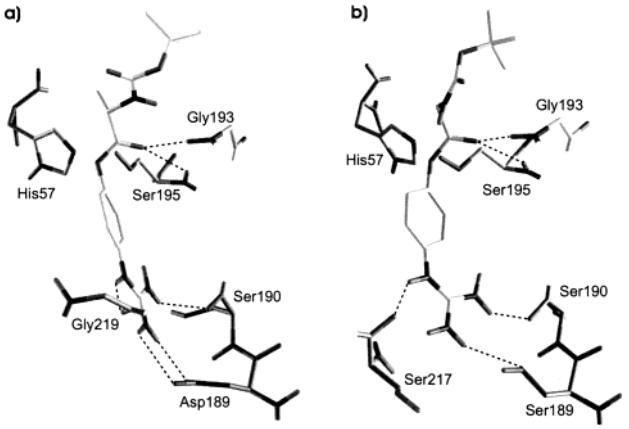
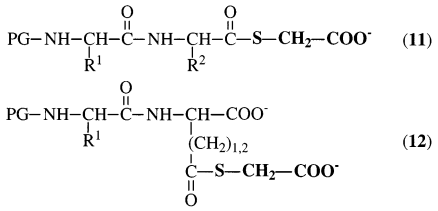


Figure 10. Arrangements of Boc-L-Ala-OGp in the active-sites of trypsin (a) and chymotrypsin (b) derived from the lowest-energy complexes (according to ref 218). Shown are the amino acid residues of the enzymes which either form hydrogen bonds (Asp189/Ser189, Ser190, Ser217/Gly219) or have catalytic functions (His57, Asp102, and Ser195).

trypsin. Although based on a different mode of individual enzyme−substrate interactions, a similar arrangement of the substrate into the active-sites of the two enzymes became evident from which the acceptance of OGp esters by chymotrypsin was suggested. Finally, this prediction was proven by model peptide syntheses using Boc-Ala-OGp and several other Boc-L/D-Xaa-OGp esters leading to the data listed in Table 6. In closing, mediated by this approach chymotrypsin (and the other proteases mentioned above) can be used as an efficient biocatalyst for coupling a wide spectrum of different carboxyl components far away from its original native specificity which is unfeasible with classical donors. Furthermore, due to the acceptance of 4-guanidinophenyl esters by trypsin and proteases with trypsin-like specificity, this type of substrate mimetics becomes likewise universal for numerous proteases. Also, these findings illustrate that it is possible on the basis of a rational approach to estimate a priori whether a substrate mimetic becomes accepted by a protease. Further investigations may show whether the docking approach could lead to novel types of substrate mimetics with more specific binding behaviors and higher acylation rates.

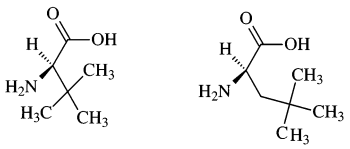
Besides synthesizing linear peptides, the substrate mimetics approach also allows for the synthesis of isopeptides.[219] The key feature of this activity is the use of a novel iso-type of substrate mimetics that directs the intrinsic synthesis activity of the protease

Scheme 6. General Structures of Classical Linear (11) and New Iso-Type (12) Substrate Mimetics[a]



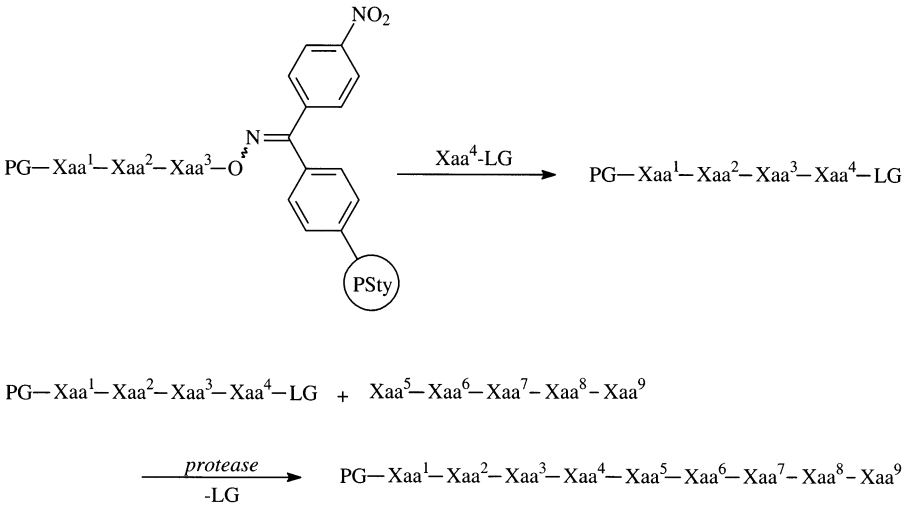
[a] The site-specific carboxymethyl thioester group is emphasized by bold letters. PG, protecting group; R^1, R^2, individual side-chains.

to the side-chain carboxyl moiety of Asp and Glu (Scheme 6). Similar to classical substrate mimetics (**11**), the novel type donors (**12**) bear a site-specific ester leaving group that mediates the acceptance of nonspecific acyl moieties by the protease. To direct the activity of the enzyme to the side-chain, the specific ester group, however, is linked to the ω-carboxyl moiety of Asp and Glu instead of being at the C-terminus of the peptide donor. This different architecture was shown to lead to a shift in the synthetic activity of V8 protease from the C^{α}-carboxyl group toward the side-chain moiety of the two amino acids finally resulting in the synthesis of isopeptides.



Utilization of the substrate mimetics approach to the synthesis of longer polypeptides indisputably needs a combination with chemical methods primarily with solid-phase peptide synthesis to provide a synthetical way to long-chain substrate mimetic reactants. Recently, this issue became initially addressed by using the oxime resin strategy.[220] While earlier work established the general applicability of this methodology for peptide ester synthesis,[221] we investigated its usefulness for the preparation of various substrate mimetics. The general synthesis protocol involves attachment of the first N^{α}-Boc-protected amino acid to the oxime resin, acetylation of free oxime hydroxylic groups, deprotection of the N^{α}-amino group of the attached amino acid, and following in situ coupling of the next N^{α}-Boc-protected amino acid. Finally, the peptide esters were generated by aminolysis of the oxime ester linkage using the appropriate N^{α}-deprotected amino acid

Scheme 7. General Course of the Combined Solid-phase Peptide Synthesis Substrate Mimetic Fragment Condensation Approach: Generation of the Donor Peptide Fragment by Aminolysis of the Oxime Ester Linkage Using an Amino Acid Substrate Mimetic, and Protease-Catalyzed Fragment Condensation[a]



[a] According to ref 220. PG, N^{α}-protecting group (Boc); LG, site-specific leaving group.

esters (Scheme 7). By utilizing this synthesis strategy, several peptide esters in form of different substrate mimetics, e.g., carboxyethyl thioesters or 4-guanidinophenyl esters, were synthesized in yields of 50% or higher. After a final deprotection step, the peptide esters have served as carboxyl components for trypsin-, V8 protease-, and chymotrypsin-catalyzed fragment condensations. Alternatively, longer chain substrate mimetics can be easily prepared by Fmoc solid-phase peptide synthesis utilizing the alkanesulfonamide safety-catch resin strategy (unpublished results). Release of the peptide esters from the resin can be achieved by activation of the resin-peptide linker following alkylation of the sulfonamide, e.g., with iodoacetonitrile. Aminolytic or thiolytic cleavage of the resulting activated *N*,*N*-cyanomethylacylalkanesulfonamide ester linkage using the respective amino acid esters or thiols yield the substrate mimetics ready for enzymatic ligation. Without doubt, the possibility of using standard synthesis methods for the preparation of substrate mimetics should further improve their synthetic value in particular for the ligation of longer chain peptide fragments.

A serious drawback of substrate mimetics became evident when peptide reactants were used which contain enzyme-specific amino acid moieties. In such instances, unwanted cleavages of those peptides as well as their related products have been found.[222] A radical shift in enzyme preferences was recently observed in frozen aqueous media. Under this condition, a number of enzymes exclusively catalyzed the coupling of the substrate mimetics with the appropriate peptide fragments remaining devoid of any proteolytic side reactions as shown in Table 7.[222] Also, freezing of the reaction mixture reduced the rate of competitive hydrolysis of the substrate mimetics which finally favored the formation of the peptide product. Hence, the combination of substrate mimetics and freezing strategy achieves irreversible and efficient peptide bond formation without the risk of unwanted proteolytic side reactions. It should be noted that similar improvements can also be reached by manipulating the catalyst itself (section B).

Table 7. Influence of Freezing on Substrate Mimetics-Mediated Couplings of Specific Amino Acid-Containing Peptides Catalyzed by Trypsin, Chymotrypsin, and BL-GSE[a]

		yield (%)	
protease/ acyl donor	acyl acceptor	25 °C	−15 °C
	Trypsin		
Bz-Gly-OGp	AA<u>K</u>AG	51.5 (85.9)	97.3
Bz-Gly-OGp	AA<u>R</u>AG	27.0 (85.4)	97.6
Bz-Glu-OGp	AA<u>K</u>AG	54.9 (76.5)	90.1
Bz-Glu-OGp	AA<u>R</u>AG	30.4 (79.4)	92.1
	Chymotrypsin		
Bz-Gly-OPh	AA<u>F</u>AG	74.1 (84.0)	90.6
Bz-Gly-OPh	AA<u>Y</u>AG	15.0 (79.5)	96.7
Bz-Glu-OPh	AA<u>F</u>AG	73.6 (83.1)	88.0
Bz-Glu-OPh	AA<u>Y</u>AG	15.0 (84.7)	94.6
	BL-GSE		
Z-Ala-SCm	AA<u>D</u>AG	31.6 (31.6)	71.8
Z-Ala-SCm	AA<u>E</u>AG	0 (34.6)	71.9

[a] According to ref 222. The specific amino acid moieties within the nucleophilic peptides are underlined. Brackets generally indicate competitive cleavages. The data in brackets correspond to the whole peptide products while the data outside give the maximum yields of the appropriate intact hexapeptide product. Conditions at 25 °C: 0.1 M HEPES-buffer (pH 8.0), 0.2 M NaCl, 0.02 M $CaCl_2$, [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-7} M, [chymotrypsin] = 1.0×10^{-6} M, [BL-GSE] = 3.0×10^{-5} M. Conditions at −15 °C: distilled water (pH 8.0 before freezing), [acyl donor] = 2 mM, [acyl acceptor] = 15 mM, [trypsin] = 1.0×10^{-5} M, [chymotrypsin] = 1.0×10^{-5} M, [BL-GSE] = 1.0×10^{-8} M.

As mentioned earlier, the direct coupling of non-proteinogenic carboxyl or amino components usually drop the efficiency of syntheses or results in a complete loss of enzyme activity under normal conditions. For the carboxyl counterpart, the use of substrate mimetics circumvents this specificity problem as recently demonstrated by our own work. Utilizing model esters of 4-phenylbutyric acid (Pbu-OGp), benzoic acid (Bz-OGp), and Bz-β-Ala-OGp as the donor, various amino acid amides and peptides as the

acceptor, and clostripain and chymotrypsin as the catalyst, the appropriate isosteric peptide products could be obtained in yields between 76 and 98% (cf. chapter IV).[218,223] Importantly, the efficiency of synthesis was practically unaffected by the disparate nonspecific acyl residue. Without any doubt, this remarkable activity opens up new possibilities to the synthesis of a broad spectrum of peptide isosteres in an easy way and under extraordinary mild conditions unachievable with classical approaches.

In recent works, we used the substrate mimetics approach and the unique specificity of clostripain toward the amino component[224] to achieve novel interesting reactions partly complete outside of peptide synthesis. One of them used 4-guanidinophenyl esters of benzoic acid and 4-phenylbutyric acid and a large number of non-amino acid-derived amino components, such as aliphatic and aromatic amines, amino alcohols, non-α-amino carboxylic acids, and symmetric and asymmetric diamines, to achieve highly selective syntheses of non-amino acid-derived carboxylic acid amides.[223] Remarkably, most enzymatic reactions have led to product yields higher than 70% (cf. chapter IV). These results become even more impressive since only traces of the amino components are deprotonated at the pH value of 8.0, which was required because of the lower intrinsic stability of the acyl donor esters. Therefore, only a small amount of the whole amine can serve as the deacylating component. Another account reports on an efficient method for the synthesis of a wide variety of *N*-linked neo-peptidoglycans and carbohydrate-containing amino acid building blocks by exploiting clostripain as the catalyst and both normal linear and iso-type substrate mimetics. In fact, the approach allows selective coupling of carboxylate moieties derived from Asp, Glu, and the C-terminus of peptides with both simple monomeric and highly complex carbohydrates, such as D-glucosamine, D-galactosamine, muramic acid, and moenomycin A, under mild aqueous reaction conditions with yields ranging between 24 and 73% (cf. chapter IV).[225]

C. Enzyme Engineering

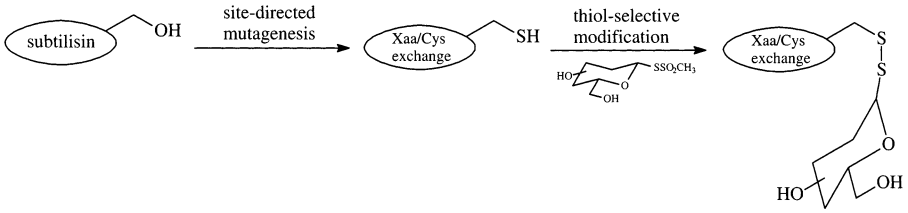
Enzyme engineering[226] is defined as a range of techniques from deliberate chemical modification to remodeling a wild-type enzyme by gene technology, while proteases are historically as well as presently at the forefront of this research. Generally, four primary goals are mainly pursued when engineering a protease for synthesis. First, one needs to make the enzyme more stable catalyst especially toward organic solvents. Improved stability in a synthetic sense usually means avoidance of irreversible enzyme inactivation under the conditions required for synthesis. Inactivation however can be a highly complex process involving unfolding, autodigestion, aggregation, or chemical damage to certain amino acids of the enzyme. Hence, the individual rate determining step of inactivation under the specific conditions must be known for applying rational approaches to enzyme stabilization. Furthermore, an enzyme species which has been stabilized toward hydrophilic organic solvents might not be inevitably more stable in hydrophobic organic media, for example. Thus, the manner in which stabilization can be achieved is often case specific although some general rules already exist.

Second and usually more difficult to accomplish, one needs to improve the synthesis efficiency of the enzyme mainly by reducing the rate of competitive hydrolysis of the acyl donor ester especially when water is used as the reaction media. Unfortunately, the molecular basis which controls the efficiency of deacylation of the acyl enzyme intermediate by water is only poorly understood. Hence, there is no general approach presently that would allow for direct suppression of competitive hydrolysis reactions by enzyme engineering. Currently, optimization the active-site specificity for better binding the acyl acceptor components remains as the only practical way to influence the ratio between product formation and undesired hydrolysis of the acyl donor. Third, one needs to reduce the usually unwanted proteolytic activity of proteases to prevent competitive peptide cleavages during the synthesis reaction. Several approaches have been developed and a few of them were found to be synthetic useful. Nevertheless, practically all the resultant enzyme variants suffer from significantly lower overall enzyme activity making it questionable whether they will reach industrial application in large-scale synthesis. Fourth, one needs to extend or alter the native enzyme specificity or enantionselectivity of the enzyme to fit them to the requirements of synthesis. As for all other goals, both chemical modification and mutagenesis can be used to create biocatalysts with the desired function different from that of the original parent enzyme. The most important and recent ones will be reviewed in the following section.

1. Chemical Enzyme Modifications

Covalent chemical modifications of enzymes can be considered as the original method available for altering enzyme properties. This relatively long history however does not mean that this kind of basic enzyme engineering has lost anything of its attraction. In fact, chemical approaches have now reemerged as a powerful complementary technique to site-directed mutagenesis and directed evolution for tailoring enzymes.[227] Several benefits contribute to this renaissance: (i) chemically modification is generally applicable; (ii) it is usually inexpensive and easy to perform even in large scale; and (iii) it allows for the incorporation of noncoded amino acid moieties and, thus, in principle leads to a variety of enzyme species that cannot be generated by genetic engineering. Classical approaches to chemical enzyme modification, however, often suffer from its lack of chemo- and regio-selectivity which can yield heterogeneous and irreproducible enzyme preparations. For example, enzyme immobilization, which represents by far the most common chemical approach to enzyme stabilization, usually proceeds in an uncontrolled manner. This inevitably leads to a random orientation of the enzyme on the support surface and may result in partial or total loss of enzyme activity if the site of attachment is close to the binding or active-site of the enzyme (cf. chapter III). In principle, this also

Scheme 8. Controlled Site-Selective Glycosyation of Subtilisin by a Combined Site-Directed Mutagenesis Chemical Modification Approach



holds true for most of the other classical chemical approaches. For example, preparation of methyl-chymotrypsin, -subtilisin, or -trypsin using methyl sulfonate reagents, originally directed to methylate His^{57} (chymotrypsin and trypsin) or His^{64} (subtilisin) of the catalytic triad of the enzymes, yields enzyme mixtures in which the remaining histidines of the enzyme molecules are partly or completely methylated.[228] However, due to the small size of the modifying moiety undesired effects of this random modification on the enzyme activity are only marginal. The methylated variants of the proteases are now recognized as interesting biocatalysts for kinetically controlled peptide synthesis mainly due to its loss of proteolytic activity with some of the esterase activity remaining.[228] Recently, Sekizaki et al. described the general applicability of methyl-trypsin for substrate mimetics-mediated peptide syntheses extending the synthetic utility of those enzymes.[229] Although important, the very low synthetic activity of methylated proteases however should generally hinder their practical use. Oxidation of Met^{192} in chymotrypsin leads to a much more active enzyme variant which, however, has still proteolytic activity.[230] Nevertheless, due to the improved esterase-to-amidase ratio and the higher stability of the enzyme toward basic conditions the Met^{192}-sulfoxide-chymotrypsin was found to be a useful enzyme for peptide synthesis. The preparation of seleno-subtilisin[231] and thiol-subtilisin[232] can be considered as further examples of small-size chemical modifications. The latter, which simultaneously marks the beginning of chemical enzyme engineering, represents the conversion of the active-site serine 221 of subtilisin to cysteine. As it turned out, this first alteration remains one of the most useful. Similar to methylated proteases, subtilisin S221C is catalytically wounded to the point that it will barely hydrolyze peptide bonds but turns out to be quite reactive with certain activated ester substrates.[233] Furthermore, thiol-subtilisin profits from the fact that thiol esters usually have higher aminolysis-to-hydrolysis ratios than regular oxo esters.[234] This combination of properties has made it a useful tool for peptide synthesis and transesterification reactions such as regioselective acylation of sugars.[235] It should be noted, however, that the catalytic activity of thiol-subtilisin is several orders of magnitude lower than that of the wild-type enzyme, although it is more active than the His-methylated species.[236] The reduced enzyme activity, however, may be especially problematic for syntheses that require the use of organic solvents. Seleno-subtilisin is even a much poorer enzyme than thiol-subtilisin.[231] Its synthetic application for peptide synthesis essentially needs the use of highly activated esters as the acyl donor components, but even in this case the reactions proceed very slowly. In addition, the enzyme is greatly sensitive toward oxidants. Although less suitable for peptide synthesis, this behavior has made seleno-subtilisin a useful artificial biocatalyst to mediate peroxidase-like reactions.[237] Recently, an impressive paper was published reporting on the preparation of the enzyme in large scale and its synthetic use to achieve the enantioselective reduction of racemic hydroperoxides.[238] The semisynthetic enzyme exhibited catalytic efficiency comparable to that of native peroxidases, but importantly yielded the opposite enantiomers, i.e., the *R*-hydroperoxide and *S*-alcohol. Furthermore, whereas natural peroxidase activity is limited to sterically unencumbered substrates, even tertiary hydroperoxides were accepted by seleno-subtilisin. Recently, the same strategy was applied to trypsin to generate the corresponding seleno-trypsin.[239] The enzyme exhibited good glutathione peroxidase activity and was shown to catalyze the peroxide-assisted reduction of glutathione. Although less active than the native glutathione peroxidase, seleno-trypsin is much more stable than its natural counterpart and is readily available.

Covalent multisite attachment of carbohydrate-based materials to enzyme surfaces has been published about three decades ago and has been already noted in chapter IV as an approach to stabilize enzymes in organic media. A functional different approach is represented by site-selective glycosylation of the active-site of proteases although it is based on a similar type of enzyme modification. The presence of a unique amino acid moiety which can undergo chemoselective reactions is an essential prerequisite to achieve those site-specific modifications. Hence, this approach shows some similarities to site-specific immobilization techniques discussed earlier (chapter III). Having a unique amino acid moiety within the active-site of an enzyme, however, is rather an exception than the rule. Subtilisin and carboxypeptidase Y can be considered as such rare enzymes as they contain no natural cysteines. Thus, incorporation of an artificial cysteine moiety within the active-site of those enzymes by site-directed mutagenesis creates such a unique reaction center that can be chemically modified to introduce an unnatural amino acid side-chain selectively (Scheme 8).[240] A series of recent papers published by the laboratory of Jones and co-workers continue to this initial work using subtilisin as the model enzyme.[241] In addition, besides carbohydrates a variety of other

Table 8. Effect of Active-Site Glycosylation at the Artificial Cysteine[166] at the Base of the Primary Specificity S_1 Pocket on the Substrate Tolerance of Subtilisin[a]

				yield[b] (%)				
acyl donor	acyl acceptor	product	time (h)	wt-subtilisin	S166C-1	S166C-2	S166C-3	S166C-4
Z-L-Phe-OBn	H-Gly-NH_2	Z-L-Phe-Gly-NH_2	1	92	95	93	91	95
Z-L-Ala-OBn	H-Gly-NH_2	Z-L-Ala-Gly-NH_2	5	91	85	77	92	83
Z-L-Glu-OBn	H-Gly-NH_2	Z-L-Glu-Gly-NH_2	5	62	58	65	54	67
Z-L-Phe-OBn	H-L-Ala-NH_2	Z-L-Phe-L-Ala-NH_2	24[c]	57	28	34	31	32
Z-L-Ala-OBn	H-L-Ala-NH_2	Z-L-Ala-L-Ala-NH_2	24[c]	0	15	16	22	11
Z-L-Glu-OBn	H-L-Ala-NH_2	Z-L-Glu-L-Ala-NH_2	24[c]	0	48	50	51	55
Z-D-Phe-OBn	H-Gly-NH_2	Z-D-Phe-Gly-NH_2	48[d]	0	6	8	7	8
Z-D-Ala-OBn	H-Gly-NH_2	Z-D-Ala-Gly-NH_2	48[d]	0	80	77	72	70
Z-D-Glu-OBn	H-Gly-NH_2	Z-D-Glu-Gly-NH_2	48[d]	0	63	62	64	64

[a] According to ref 245c with permission of the Royal Society of Chemistry. Copyright 2001. Conditions: DMF-water, 1:1; 0.1 M donor; 0.3 M acceptor; 0.036 mol % enzyme. [b] Isolated yields. [c] 0.2 M acceptor. [d] Further 0.036 mol % added at 24 h. S166C-1, -$(CH_2)_2$-O-β-D-Glc$(Ac)_2$; S166C-2, -$(CH_2)_2$-O-β-D-Glc$(Ac)_3$; S166C-3, -$(CH_2)_2$-O-β-D-Gal$(Ac)_3$; S166C-4, -$(CH_2)_2$-O-β-D-Gal.

structures differing in size and physicochemical properties were covalently linked to artificial cysteines mainly in position 62, 166, or 217 which correspond to the enzyme's S_2, S_1, and S_1' subsite, respectively. Subsequently, the resultant library of enzyme variants has been subjected to investigations on the influence of the individual active-site modification on the activity and specificity of subtilisin. Interestingly, remarkable effects were found on both catalytic properties. Keeping in mind that most chemical active-site modifications lower the activity of the enzyme this was not the case for this type of active-site engineering throughout. In fact, incorporation of the hydrophobic ligand -CH_2-*c*-C_6H_{11} at the enzymes S_2 subsite for example increased the activity of subtilisin more than 3-fold compared to the parent enzyme[242] probably by lowering the pK_a of His[64] of the catalytic triad.[243] Additionally, an improved ratio of esterase vs amidase activity was found for a number of enzyme variants[244] which was in some cases connected with a broader substrate tolerance as exemplarily illustrated in Table 8.[245]

Recently, Jakubke and co-workers established the use of zymogens as novel catalysts for peptide synthesis.[246] Although widely seen as inactive precursors of active proteases, zymogens were found to be slightly active toward activated peptide esters.[247] Initiated by these earlier findings Jakubke et al. investigated the synthetic utility of both trypsinogen and chymotrypsinogen for peptide synthesis.[248] To inhibit auto-activation of the zymogenes, which proceed via limited proteolysis of the Lys[15]—Ile[16] bond, trypsinogen was exemplarily guanylated on all of its 15 Lys residues chemically. The modified enzyme irreversibly fixed in its zymogen state was still active for peptide ligation, but exhibited no detectable activity for cleaving peptide bonds. Although proceeding in an irreversible fashion, the rates of the synthesis reactions were only very low, which may hinder the preparative use of this interesting approach at least without further rate improving manipulations.

2. Genetic Enzyme Modifications

The history of genetic enzyme modification is closely connected with proteases in particular with subtilisin. The first genetic modifications in this enzyme occurred rapidly after the gene was cloned in the early 1980s.[249] Nearly two decades later, mutations in well over 50% of the 275 amino acids of subtilisin have been reported in the scientific literature.[250] Additionally, a number of other proteases became targets of genetic modifications. Besides protease engineering for synthesis which mainly include manipulations of the stability, specificity, and activity, a number of other enzyme properties turned out to be subjects of genetic modifications, such as catalytic mechanism,[251] surface activity,[252] or folding mechanisms.[253] Presently, a variety of techniques exist for introducing changes into the enzyme at the genetic level. In general, all of them are useful to tailor the enzyme properties to the requirements of synthesis. Basic principles and recent examples of synthetic importance closely related to proteases will be summarized in the following section. More general reviews covering the whole field of protein engineering can be additionally found in the recent scientific literature.[254]

Site-directed mutagenesis can be considered as one of the first genetic modification techniques and has proved, in turn, to be a powerful method for dissecting relationships between enzyme structure and function. It has contributed significantly to our understanding of enzyme catalysis in general, and has also led to the development of protease variants with improved properties for synthetic transformations. Although simple to perform, it does, however, require that one have some idea of which residues are important. Having a three-dimensional structure of the enzyme in question is particularly helpful in selecting those important moieties. Due to the inability to predict long-range structural changes, most protease engineering continues to involve catalytic amino acids, substrate binding regions, and direct stabilizing mutations. With regard to peptide synthesis, subtilisin species with enhanced esterase-to-amidase ratio, modified specificity, improved stability, and altered pH profile have been designed by this method.[255] Efforts in this field, for example, led to a double mutant, i.e., so-called "subtiligase", in which the catalytic Ser[221] is exchanged with Cys and Pro[225] with Ala.[256] The enzyme variant has been used for the complete synthesis of wild type and mutant ribonuclease A in milligram quantities by stepwise ligation of six esterified peptide fragments, each 12 to 30 residues long.[257] Single and multiple site-

specific mutations have also been extensively used to obtain subtilisin variants with increased stabilities toward thermal denaturation[258] and inactivation by organic solvents.[259] Although the enzyme stability could be significantly increased in a number of instances, the successful rational design of a completely organic solvent stable protease still remains an unsolved challenge. The lack of knowledge about the molecular basis of enzyme stabilization hinders the rational design and makes the success of this approach a matter of trial and error. For example, Kidd et al. mutated a surface lysine to tyrosine in subtilisin reasoning that removing surface charges would increase stability.[260] Indeed, the stability was improved, but the following X-ray crystal analysis showed that the increase was due to an unintended change in the weak calcium binding site about 12 Å away. Similar findings were obtained from studies attempting to stabilize subtilisin by disulfide cross-linking. In fact, of 18 different disulfide cross-links that have been engineered into the enzyme, only three have increased stability.[261] Two of these stabilize exclusively in the presence of EDTA and only one engineered disulfide bond mutant has a higher intrinsic stability reflected by a lower rate of unfolding. The latter contains a disulfide between residues 61 and 98, which was modeled after a naturally occurring disulfide in aqualysin I from *Thermus aquaticus*.[261b] Although the work in this field is dominated by subtilisin, there are a number of other proteases that became targets for site-directed mutagenesis mediated enzyme optimization. For example, Martinez and Arnold report on the design of organic solvent stable α-lytic protease variants by systematically exchanging charged amino acid moieties on the enzyme surface with uncharged counterparts.[262] Studies on a bacterial neutral protease of the thermolysin type led to the identification of a surface-located region of the enzyme crucial for its stability.[263] Stabilization of this sensitive region by introducing an artificial disulfide bond resulted in a significant improvement of the thermostability of the appropriate protease variant, which had a half-life of more than 30 min even at 92.5 °C.[264] Breddam and co-workers investigated the importance of Trp^{312} on the P_1 substrate preference of carboxypeptidase Y.[265] A similar work has been published by the same laboratory studying the significance of Thr^{60} and Met^{398}, which were expected to be the major determinants of the P'_1 specificity of carboxypeptidase Y, in the hydrolysis and aminolysis of various peptides.[266] Much higher aminolysis rates were obtained with some of the mutant enzymes, presumably due to a changed accessibility of water to the acyl enzyme intermediate while the nucleophile/leaving group is bound at the S'_1 binding site. Elliott et al. report on the mutagenic substitution of the active-site serine residue of *Streptomyces griseus* protease B (SGPB) by either glycine or alanine.[267] Conversions of this type have been already performed in earlier studies for other serine proteases, such as subtilisin[268] and trypsin,[269] and typically led to nearly complete enzyme inactivation. Interestingly, in the case of SGBP the decrease in enzyme activity was less pronounced (about 4 orders of magnitude for the Ala mutant compared to the wild type enzyme) enabling the use of this mutant enzyme for analytical-scale peptide synthesis. Although the enzyme variants contain no active serine, a reaction mechanism proceeding through an acyl enzyme intermediate seems to be likely whereas the active serine is probably substituted by His^{57} of catalytic triad.

Site-directed mutagenesis had also led to several trypsin variants with improved synthetic properties for substrate mimetics-mediated peptide syntheses. In one report, the trypsin variant Asp189Ser was used as the starting point for the stepwise optimization of the catalyst.[270] Guided by computational docking studies, further amino acid moieties of trypsin's specificity determining S_1 binding pocket which may be responsible for undesired proteolytic side reactions were identified and subsequently exchanged with alanine. The resultant trypsin D189A,S190A double mutant exhibited an improved synthetic utility with practically no cleavage activity toward originally trypsin-sensitive peptide bonds, but unfortunately it was somewhat active for cleaving peptide bonds succeeding tyrosine. In another study, we exchanged Asp189 of trypsin with Glu.[271] As a result of this active-site mutation, the substrate preference of the protease shifted from Lys and Arg to OGp-type substrate mimetics and, therefore, from the cleavage of Lys-/Arg-bonds to the substrate mimetics-mediated synthesis of peptide bonds (Figure 11). However, some proteolytic side reactions remained as illustrated by Figure 11. Hence, to make full practical use of trypsin for substrate mimetics-mediated peptide synthesis further enzyme optimization will be required to accomplish a better differentiation between the specificity constants for the substrate mimetics and for specific peptide bonds. In closing, enzyme engineering by site-directed mutagenesis continues to be useful for improving proteases for synthesis. Further improvements of this technology can be expected from a better understanding of the relationship between the structure and the function of enzymes and from sequence and structure alignments between related enzymes. However, the longstanding inability to predict the exact protein structure required for the stereoselective reaction of a given substrate or which is necessary for high stability and activity of the enzyme in a given reaction media still remains presently as one of the main hindrances associated with this technology. Consequently, specific changes made to a protein will not inevitably have the effects expected and may easily lead to false explanations without structure proof.

Recently, *in vitro-directed evolution* using random genetic mutation and recombination has been explored as a more generally applicable approach to the modification of enzyme properties, such as enzyme activity, stability, and specificity.[254] This technology has the advantage that is does not require a priori knowledge of the relationship between enzyme structure and function. Ideally, enzymes undergoing directed evolution should be directed into the extracellular medium to facilitate enzyme separation.

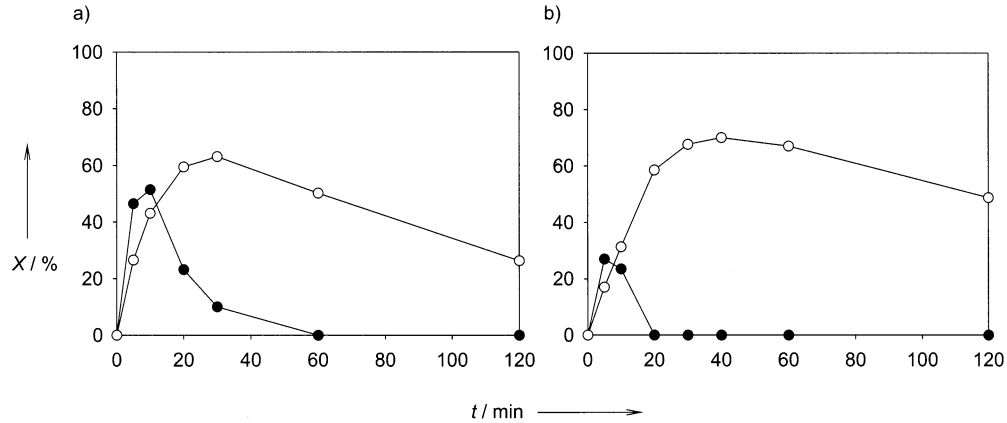


Figure 11. Time-course of the *wt*- and mutant trypsin D189E-catalyzed formation and degradation of Bz-Gly-Ala-Ala-*Lys*-Ala-Gly-OH (a) and Bz-Gly-Ala-Ala-*Arg*-Ala-Gly-OH (b) according to ref 277. (-●-) *wt*-trypsin, (-○-) mutant trypsin D189E. Conditions: 0.2 M Hepes buffer, pH 8.0, 0.2 M NaCl, 0.02 M $CaCl_2$, 25 °C, 10% methanol, [Bz-Gly-OGp] = 2 mM, [Ala-Ala-(*Lys*/*Arg*)-Ala-Gly] = 15 mM, [*wt*-trypsin] = 1.0×10^{-7} M, [trypsin D189E] = 3.1×10^{-6} M, *X* = product yield.

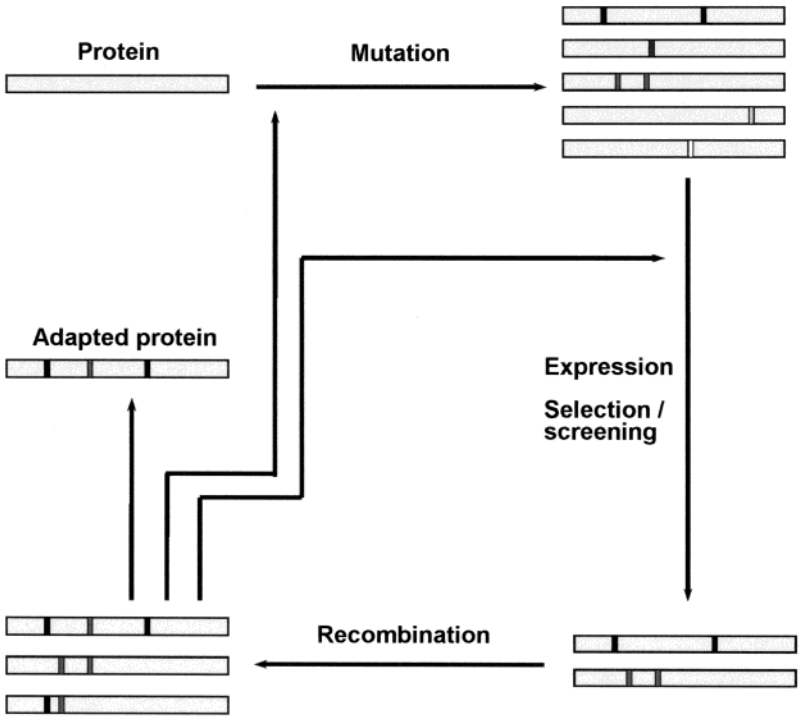


Figure 12. Strategy for the creation of new enzymes by in vitro directed evolution.

Once identified, the genes encoding the improved enzymes can be subjected to iterative rounds of mutagenesis, screening, and selection until a desired level of improvement has been reached (Figure 12). Random mutations can be introduced into an enzyme in various ways, including chemical mutagens, mutagenic base analogues, error-prone PCR, and spiked synthetic oligonucleotides. The key elements to any directed evolution project is a powerful expression system for the enzyme of interest and a fast screening or selection technique as well. Furthermore, the improvement steps during directed evolution can be less than a factor of 2, so screening requires a highly accurate method.[272] Therefore, the development of efficient screening methods significantly contributes to this research. Recent efforts in this context, for example, are published by Janes et al. who developed a fast method for measuring the enantioselectivity of hydrolases that may be useful for screening mutants with improved enantioselectivity.[273] Restrictions are given by the number of mutants that can be examined routinely. Recently, Bryan reminded us that standard screenings are usually limited to a number of individual mutants in the range of 10^4–10^5 species.[250] All combinations of double substitutions in subtilisin, for example, would yield a total of 3×10^7 different variations. Accordingly, only the population of single mutations can be adequately searched for appropriate events. However, even this population becomes further reduced by the degeneracy of the genetic code. In fact, only about 30% of the possible single substitution mutants would be produced from single base substitutions. Additionally, the method itself which is used to create mutations influences the number of individual mutants. For example, by error-prone PCR an average of only 5.7 amino acid substitutions is accessible for any given amino acid residue.[274] Thus, the diversity of the

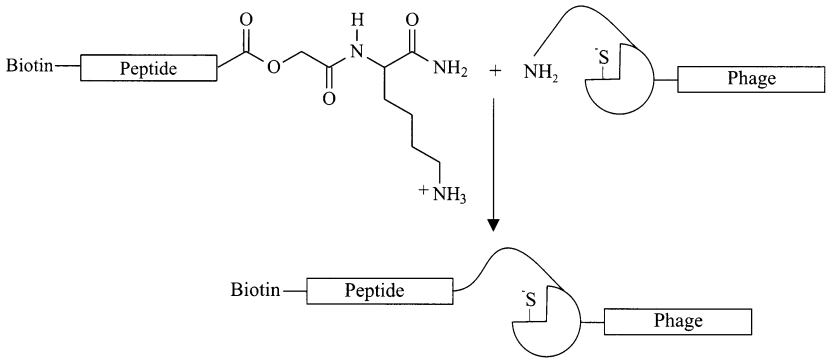


Figure 13. Scheme for selecting active subtiligase variants on phage by requiring that the enzyme attach a biotin-labeled peptide onto its extended *N*-terminus. The biotin-labeled subtiligase phages are then catured with immobilized neutravidin (according to ref 284 with permission of the National Academy of Sciences. Copyright 1999).

population is often smaller than widely expected, which explains why only mutants very close to the structure of the parent enzyme will be usually found. As a result, purely random evolution of a biocatalyst is especially useful to improve more global (cumulative) properties, such as stability, rather than the native activity or specificity of an enzyme, which usually depends on synergistic mutational events. Recent studies on the improvement of protease stability came, for example, from Sattler et al.[275] and added to a list of earlier studies in this field.[276] The authors randomly mutated subtilisin and successfully screened for enhanced thermostable variants by temperature-gradient gel electrophoresis. A more recent study of Zhao and Arnold[277] gave an impression of the number of accumulating point mutations that can contribute to an increase in stability. In total, 11 stabilizing mutations were identified while a number of them could not have been predicted using rational design approaches. Further stabilization of the enzyme could be reached by targeting random mutagenesis to positions at which stabilizing changes were already found.[274] Straussberg et al. report on a similar combination of design and random mutagenesis which enabled the creation of a hyperstable calcium-free subtilisin.[278] Investigations on its stability revealed that it inactivates 250 000 times more slowly than the wild-type enzyme in 10 mM EDTA. Also, efforts in stabilizing the activity of subtilisin in aqueous−organic media continue to be successful. On the basis of earlier attempts,[279] the laboratory of Arnold created a subtilisin variant containing 10 substitutions as a result of five sequential rounds of random mutagenesis and two additional substitutions by site-directed mutagenesis.[280] Subsequent hydrolysis studies verified an increase in enzyme activity by 256-fold in 60% aqueous DMF, while structure analysis showed that all 10 substitutions were located around the active-site. Further improvements of the mutated subtilisin in DMF could be reached by introducing three additional substitutions by random mutagenesis.[281] The resultant subtilisin variant having 13 substitutions in total has been shown to have 471 times higher hydrolytic activity in 60% DMF than the original parent enzyme. Interestingly, in all instances the enzymes optimized toward DMF were less active in purely aqueous media.

Genetic modifications can also be introduced in the gene of interest by *phage display* methods originally developed by Smith.[282] Although less direct than purely in vitro methods, phage display selections have the benefit of an increased number of mutants that can be screened.[283] Commonly, more than 1×10^9 independent variants are covered by this technique, which allow, for example, screening all combinations of amino acids at six specified positions of subtilisin. Although an obvious limitation of phage display is that selection is achieved by binding activity, several reports on using this technology for improving the catalytic behavior of enzymes exist in the scientific literature. Relating to proteases, in one case random mutations at 25 positions within the active-site have been introduced into subtiligase to select for increased peptide ligation activity.[284] Screening of improved candidates was achieved with the help of a biotin-tagged peptide that allowed for product capture at the catalyst's own extended N-terminus (Figure 13). Two new mutants, i.e., M124L,-L126V and M124L,S125A, were identified that increased the activity of subtiligase by 2.5- and 2.7-fold, respectively. Interestingly, many other mutants were selected that likely improved stability and resistance to oxidation, as these very same mutants are known to improve these functions in subtilisin. Additionally, a number of others for improving functional enzyme display were selected. In a second study, Legendre et al. displayed fully active subtilisin on the phage surface with the aim to change the S_4 specificity of the native enzyme.[285] The addition of a reversible inhibitor to the culture medium was found to be essential to avoid uncontrolled proteolysis by the enzyme. Accordingly, the selection for a change in S_4 specificity was performed using biotin-tagged peptide ester inhibitors.

Another efficient way to create new molecular diversity is through recombination of related genes. So-called *DNA-shuffling* methods pioneered by Stemmer[286] mimics the process of natural recombination generating highly diverse sequences, but conserves enzyme function. This technique involves the recombinative exchange of small DNA fragments between two or more related parent genes (homologous recombination) leading to the creation of hybrid gene libraries. Also, genes from multiple parents and even from different species can be shuffled in a single step

(DNA family shuffling or molecular breeding), operations that are forbidden in nature.[287] Recently, an impressive example of the DNA-shuffling approach related to proteases was published by Ness et al.[288] The authors started from a family of 25 subtilisin gene sequences and shuffled them with a wild type subtilisin gene. The resulting library of the corresponding enzymes then was tested for thermostability, solvent stability, activity, and pH dependence. Multiple clones were identified that were significantly improved over any of the parental enzymes for each property tested.

New biocatalysts can also be constructed from originally noncatalytic molecules. Due to the inability to the de novo design of enzymes, a suitable scaffold is needed in which an enzymatic activity can be modeled. Attempts related to the creation of those biocatalysts bearing protease-like synthesis activity are mainly focused on *catalytically active antibodies*. Research in this field was pioneered by the laboratory of Hirschmann who could show that antibodies can be programmed to catalyze peptide bond formation.[289] Recently, an extension of this approach to the synthesis of cyclic peptides has been reported by the same laboratory.[290] Starting from the *p*-nitrophenyl ester of the hexapeptide D-Trp−Gly−Pal−Pro−Gly−Phe (Pal, 3-pyridylalanine, which was used instead of the initial Phe[3] to improve the solubility), they could demonstrate that the so-called antibody ligase 16G3 catalyzes head-to-tail cyclization to give *c*-(D-Trp−Gly−Pal−Pro−Gly−Phe). The rate enhancement for the antibody-mediated cyclization was found to be 22-fold compared to the background reaction; in other words, in absolute terms, 1 μM of antibody active-sites form 2 μM of cyclic products per minute. This catalytic activity was sufficient to form the desired cyclic peptide in greater than 90% yield. Interestingly, neither epimerization nor hydrolysis of the peptide ester could be detected. Substitution of the amino acid residues of the hexapeptide at the coupling positions (positions 1 and 6) by Trp or D-Phe (position 1) and D-Phe or Trp (position 6) significantly reduces the rate of enhancement. This finding reflects the extremely high, hapten-induced specificity of the antibody. Hence, the antibody ligase acts rather as a template to channel the activated linear peptide ester into formation of the desired cyclic product than as a common enzyme (Figure 14). On the other hand, it can be expected that the length and composition of the linear peptide may be not a limitation for antibody catalysis because antibodies can be tailor-made to recognize those particular side-chains that are involved in the ring closure. However, due to the relatively low catalytic efficiency of such reactions, it is questionable whether antibodies can reach practical relevance soon.

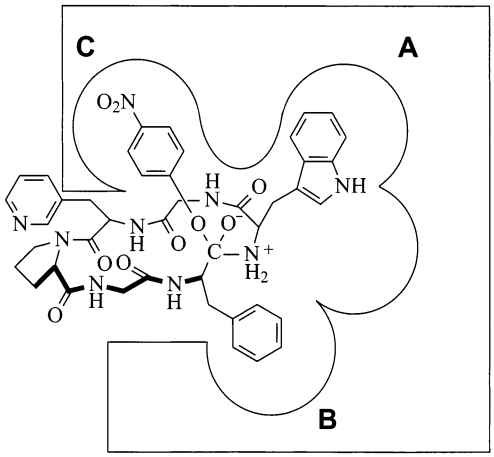


Figure 14. Representation of the proposed tetrahedral intermediate for the 16G3-catalyzed cyclization of a linear hexapeptide according to Smithrud et al. (according to ref 290 with permission of the National Academy of Sciences. Copyright 2000). Only the diastereomer containing L-Phe and D-Trp will bind to pockets A and B optimally. The remaining four amino acids are presumed to reside largely outside the binding pocket.

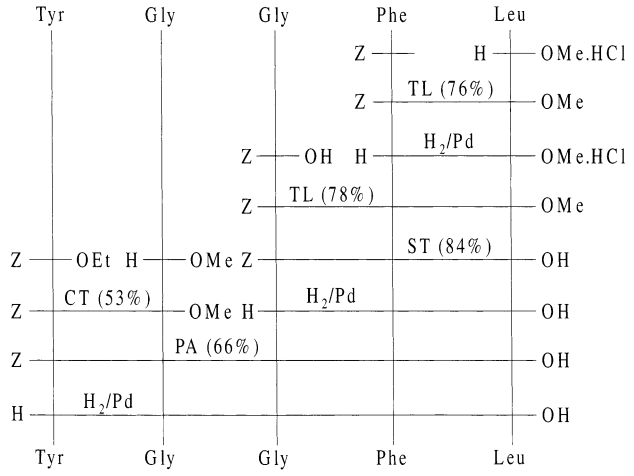
IV. Synthetic Applications

The long history of proteases as useful catalysts for organic synthesis does not mean that these enzymes have lost anything of their attraction. In fact, a number of researchers turned their attention to finding new synthetic applications or to improving existing ones. The reasons for this success arise from the commercial availability of these enzymes at mostly reasonable cheap prices, their synthetic flexibility and stability, and the ease of their handling. The resulting large number of papers reporting on the synthetic use of proteases, however, makes it quite impossible to cover all reactions performed, so the examples presented are necessarily selective. The reactions selected are those which, in the opinion of the author, have general applicability or particular significance. Guidance through the following sections is aided by headings classifying the individual reactions presented to the corresponding superordinate reaction type.

A. Peptide Synthesis

Despite the important progress that has been made in the improvement of proteases for synthesis their use as standard catalysts in peptide chemistry still remains the exception rather than the rule. Reasons are the lack of a general approach that could be routinely used. Time-consuming optimization and the necessity of having detailed knowledge on the enzyme's specificity, activity, and stability are often essential prerequisites for successful synthesis. Hence, it is not surprising that the synthesis of short peptides still dominates that of larger ones. Recent examples are the synthesis of a series of histidine-containing dipeptides catalyzed by trypsin and chymotrypsin;[291] the formation of several dipeptides by a new thermophilic protease from *Clostridium thermohydrosulfuricum*;[292] the pepsin-catalyzed synthesis of various model peptides;[293] the use of Pronase from *S. griseus* as a catalyst for dipeptide synthesis;[294] the preparation of short peptides such as, e.g., kyotorphin (H-Tyr−Arg-OH) starting from H-Tyr-OEt and Arg-OH[295] or H-Asp−Phe−Ala−Leu-OH by condensation of H-Asp−Phe-OMe and H-Ala−Leu-OH,[190] and various peptide esters in frozen aqueous media;[296] the application of prolyl endopeptidase from *Flavobacterium meningoseptum* for model peptide synthesis;[297]

Scheme 9. Synthetic Scheme of the Preparative Scale Leu-Enkephalin Synthesis in a Solid-to-Solid System[a]

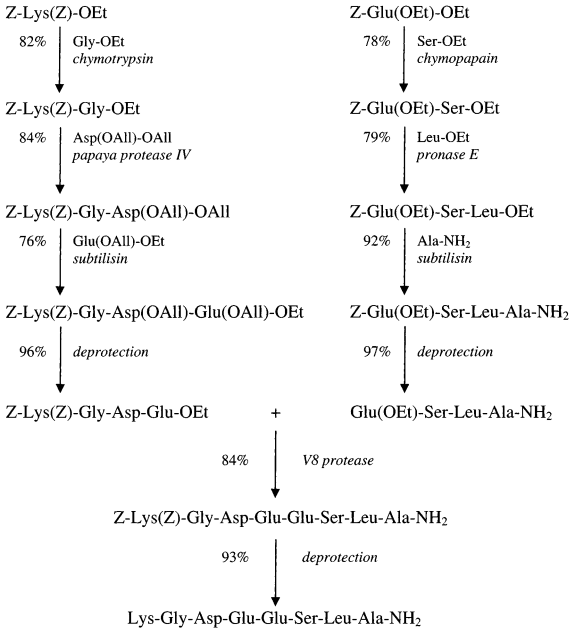


[a] According to ref 299 with permission from Blackwell Science Ltd. Copyright 1996. The syntheses were carried out in a rotary glass homogenizer by admixing solid reactants with native proteases and $Na_2CO_3 \times 10\ H_2O$ as the water source. CT, chymotrypsin; PA, papain; ST, subtilisin; TL, thermolysin.

the carboxypeptidase A-catalyzed formation of dipeptides in various water-miscible organic solvents;[298] and the thermolysin-catalyzed preparation of the aspartame precursor Z-Asp−Phe-OMe in a solid-to-solid system.[208] Additionally, a large number of other reactions that were performed within systematic or mechanistic studies are reported throughout this review.

Furthermore, a few papers have been published on the stepwise synthesis of shorter oligopeptides using different proteases as the coupling reagents. For example, Klein and Cerovsky published an interesting fully enzymatic synthesis of Leu-enkephalin by applying four different proteases in a solid-to-solid system (Scheme 9).[299] Another example was published by Gill et al. who report on the convergent (4+4) synthesis of the highly functionalized "delicious octapeptide" Lys−Gly−Asp−Glu−Glu−Ser−Leu−Ala-NH_2 with an overall yield of 39%.[300] As illustrated by Scheme 10, amino acid esters, which can act as both acyl donors and acyl acceptors, were used as the reactants for carrying out chain extension in a continuous and repetitive manner. Discrimination between the acyl donor and acceptor has been achieved by the enzyme selectivities enabling the synthesis in a highly controlled way. Finally, the N-terminal Lys moiety was deprotected following the V8 protease-mediated ligation of the two stepwise synthesized tetrapeptides. A similar combination of stepwise and convergent synthesis strategy has been used for the total synthesis of Leu- and Met-enkephalin derivatives in organic low-water systems at a preparative scale.[301] Chymotrypsin, papain, thermolysin, and bromelain adsorbed on Celite were used as the biocatalysts. Both syntheses have been carried out in four enzymatic steps and one or two chemical deprotection steps resulting in overall yields of the desired products between 40 and 54%. Although successful, the usually low activity of proteases to acylate amino acid esters and the risk of the formation of oligomeric side products are general drawbacks that may hinder the universal use of this synthesis strategy. An alternative convergent approach that circumvents these general disadvantages was used by Ye et al. for the synthesis of N^{α}-Z-protected Leu-enkephalin (Z-Tyr−Gly−Gly−Phe−Leu-OH).[13] The authors started from N^{α}-Z-protected tyrosine ethyl ester and the dipeptide ester H-Gly−Gly-OEt which were ligated by chymotrypsin in dichloromethane. In parallel, Boc-Phe-OH has been coupled with H-Leu-NHNHPh (NHNHPh, phenyl hydrazine) using thermolysin as the catalyst and *tert*-amyl alcohol as the solvent. Finally, the two synthesis products Z-Tyr−Gly−Gly-OH and H-Phe−Leu-NHNHPh were ligated under thermolysin catalysis in *tert*-amyl alcohol. The combined application of the kinetically and equilibrium-controlled approach in organic media enabled the synthesis of the Leu-enkephalin derivative in an overall yield of about 30%.

Scheme 10. Protease-Catalyzed Convergent (4+4) Synthesis of the "Delicious Octapeptide" Lys−Gly−Asp−Glu−Glu−Ser−Leu−Ala-NH_2[a]



[a] According to ref 300 with permission from the American Chemical Society. Copyright 1995.

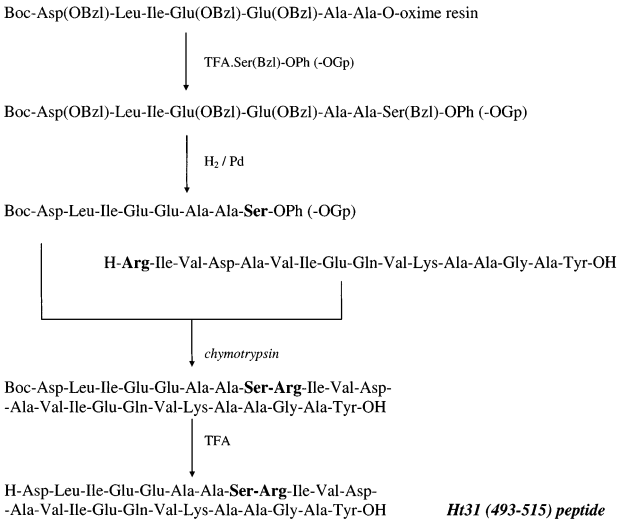
Without considering purely chemical strategies, oligopeptides exceeding the range of 5 or 8 amino acids in length are best synthesized in a nonstepwise manner by the ligation of chemically prepared peptide fragments. As a result of our attempts to improve and simplify the existing classical enzymatic methods, we combined the substrate mimetics strategy with the oxime resin approach to achieve the semisynthetic preparation of the biologically active 493−515 sequence of human thyroid PKA (protein kinase A)-anchoring protein Ht31.[302] The synthetic route to the final Ht31 peptide is illustrated in Scheme 11. The carboxyl component Boc-Asp−Leu−Ile−Glu−Glu−Ala−Ala−Ser-OGp used as a substrate mimetic has been synthesized via oxime resin strategy, while the hexadecapeptide was prepared by standard Fmoc-solid-phase peptide chemistry. For chymotrypsin-

Table 9. Yields (%) of the V8 Protease-Catalyzed Synthesis of Isopeptides[a]

	acceptor peptide				
donor peptide	H-Met-NH_2	H-Gly−Leu-NH_2	H-Leu−Gly-NH_2	Ile-Ala−Ala−Ala-Gly	Leu−Ala−Ala−Ala-Gly
Z-Glu/*Asp*(SCm)-OH	41.2/*41.5*	40.3/*41.6*	47.7/*48.5*	49.8/*49.3*	50.5/*52.2*
Z-Ala−Glu/*Asp*(SCm)-NH_2	41.3/*59.5*	39.8/*56.9*	47.0/*60.3*	47.2/*67.9*	50.3/*65.9*
Z-Ala−Ala−Glu/*Asp*(SCm)-NH_2	44.9/*53.9*	45.1/*56.6*	50.4/*61.9*	49.4/*62.3*	51.0/*61.8*
Z-Glu/*Asp*(SCm)-Ala-NH_2	39.0/*51.7*	39.6/*54.2*	46.5/*59.7*	48.6/*66.8*	49.3/*64.3*
Z-Glu/*Asp*(SCm)-Ala−Ala-NH_2	44.7/*58.7*	45.4/*55.8*	52.6/*61.7*	54.6/*67.5*	54.9/*64.8*
Z-Ala−Glu/*Asp*(SCm)-Ala-NH_2	42.5/*60.9*	43.9/*62.8*	50.2/*65.7s*	50.1/*67.4*	51.6/*68.0*
Z-Ala−Ala−Glu/*Asp*(SCm)-Ala−Ala-NH_2	40.8/*59.1*	41.5/*61.9*	51.9/*64.0*	52.4/*66.4*	52.8/*65.0*

[a] According to ref 219. Conditions: 0.2 M HEPES buffer, pH 8.0 at 37 °C; [donor peptide] = 2 mM, [acceptor peptide] = 15 mM, [V8 protease] = 3.61−10.33 μM; reaction time: 5−20 h.

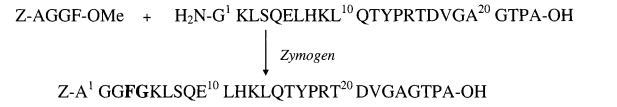
Scheme 11. Scheme of the Semisynthesis of the Ht31 (493−515) Peptide on the Basis of the Enzymatic Fragment Condensation via Substrate Mimetics Strategy[a]



[a] According to ref 302.

mediated peptide ligation the best yields were achieved when the molar ratio of carboxyl to amino component was 2:1. Under these conditions, the complete consumption of both peptide fragments accompanied by the formation of the N^{α}-Boc-protected Ht31 peptide product could be achieved. Finally, TFA-treatment led to the nativelike Ht31 peptide which was further used in biological assays to study the PKA-dependent regulation of ion channels in mammalian heart cells.[303] Also, a combination of solid-phase peptide synthesis and protease-mediated fragment ligation was used for the synthesis of native and mutated RNase A.[257] As mentioned earlier (cf. section C. Enzyme Engineering) the synthesis approach involved stepwise ligation of six esterified peptide fragments using subtiligase as the biocatalyst. The synthesis starts with the solid-phase synthesis of the fragment (98−124) followed by deprotection. The next fragment (77−97) was used as Phe-NH_2-modified carboxamido methyl ester. This special type of ester can be considered as fine-tuned analogues of unmodified carboxamido methyl esters which themselves have been used in other enzymatic syntheses.[304] To prevent self-ligation, the N-terminus of the peptide fragment was blocked by the isonicotinyloxycarbonyl (iNoc) protecting group. The iNoc group was incorporated at the last step of the solid-phase synthesis, is stable to anhydrous hydrofluoric acid, and can be removed under mild reducing conditions (Zn/AcOH). Since subtiligase is efficient with large and hydrophobic P_1 donor substrates and efficient with small nonpolar or positively charged residues at P_1' the ligation was determined between Tyr and Lys residues. In a similar way all following steps were performed. In another, yet unpublished account, Jakubke and co-workers evaluated the applicability of zymogens for irreversible fragment condensation.[305] In fact, the coupling reaction of a synthetic tetrapeptide with a recombinant 24-peptide was studied using commercial chymotrypsinogen as the biocatalyst (Scheme 12). The ligation reaction was performed in aqueous−organic mixture containing 40% (v/v) DMSO. After 400 min and complete acyl donor consumption 60% of the desired peptide product could be obtained.

Scheme 12. Course of the Chymotrypsinogen-Catalyzed (4+24) Fragment Condensation[a]



[a] According to ref 305 with permission from the corresponding author.

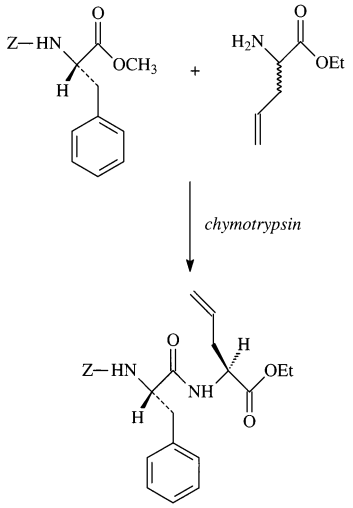
Besides the synthesis of linear peptides, proteases can also mediate the formation of isopeptides. This catalytic activity needs the use of a novel type of substrate mimetics as the acyl donor component bearing the side-specific ester leaving group at the ω-carboxyl moiety of Asp and Glu instead of being linked to the acyl donor's C-terminus (cf. section B. Substrate Engineering). This different architecture was shown to lead to a shift in the synthetic activity of V8 protease from the C^{α}-carboxyl group toward the side-chain moiety of the two amino acids finally resulting in the synthesis of isopeptides as it was shown in initial structure−function relationship studies (Table 9). Furthermore, proteases can also mediate head-to-tail cyclizations of peptides. Initially, this interesting activity was shown by Wells and coworkers using subtiligase as the catalyst.[306] In this work, peptide glycolate phenylalanylamide esters of chain lengths between 10 and 31 amino acids and with unprotected side-chains were used as the linear precursors. The researchers found that peptide esters shorter than 12 residues only hydrolyze or dimerize, but do not cyclize. In the case of the longer peptides,

a cyclization could be detected. The yields for cyclization ranged from 30 to 85% and the efficiency usually increases along with the length of the peptide. The authors suggested that longer peptides may be more flexible and, therefore, better able to adopt a productive binding conformation. Furthermore, the efficiency of cyclization appears to depend on the sequence of the precursor peptide. According to the specificity of subtiligase, the highest yields on cyclic peptides were found with large hydrophobic residues at the P_1 position (C-terminus) and small nonpolar residues at the P'_1 position (N-terminus). At the remaining positions, a variety of sequences are accepted by the enzyme, which makes this method a rather general one for the synthesis of larger cyclic peptides. In principle, peptide cyclization can also be achieved by native proteases. In a preliminary study, Burger and Bartlett investigated the capability of trypsin to catalyze the formation of amide bonds by intramolecular cyclization of resin bound peptides.[307] On the basis of the significant activity found, a novel approach to screening a library of linear compounds for enzyme cyclization has been established. A review about other enzymatic approaches to peptide cyclization was recently published.[308]

B. Synthesis of Peptide Isosteres

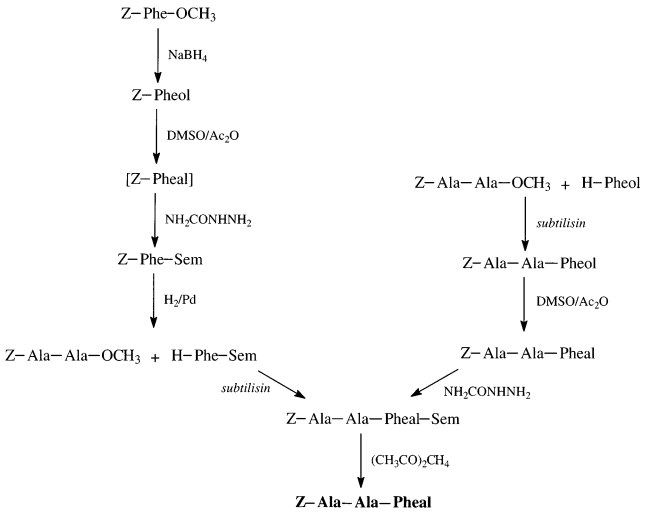
The utility of proteases for synthesizing peptide isosteres is mainly based on the flexibility of some of these enzymes to acylate not only nucleophiles derived from coded amino acids, but also those containing noncoded amino acid moieties and even non-amino acid-derived amines at the site of acylation. Nevertheless, the product yields usually drop with increasing degree of modification of both the amino acid side-chain and the backbone structure. Therefore, only the coupling of acyl acceptors closely related to preferred amino acid residues is usually of practical relevance. Broadening of the substrate specificity, however, can be achieved by manipulations of the reaction medium, the substrates, or the enzymes themselves. Recent examples for the acylation of noncoded amino acid moieties are published by Fernandez et al.[309] The authors investigated the ability of Nagarse and chymotrypsin to catalyze the formation of Z-Phe-Ag-OEt (Ag, allylglycine) starting from Z-Phe-OMe and L,D-Ag-OEt (Scheme 13). While Nagarse gave no reaction, chymotrypsin was found to be able to synthesize the desired LL-product in about 50% yield using a biphasic reaction system. A further report on the synthesis of dipeptide isosteres stems from Krix et al.[310] The authors successfully acylated *tert*-leucine (**11**) and neopentylglycine (**12**) with Z-protected phenylalanine using various proteases and reaction systems. The semi-enzymatic synthesis of a series of peptide aldehydes of the general formula Z-Ala−Ala−Xaa-al (where Xaa-al = leucinal, phenylalaninal, alaninal, and valinal) has been described by Voyushina et al.[311] As shown in Scheme 14, the desired peptide aldehydes were obtained via two distinct strategies, while in both cases immobilized subtilisin was used as the biocatalyst. Performing the enzymatic coupling reactions in a neat organic solvent (95% acetonitrile, 5% DMF (v/v)) led to product yields usually higher than 80%. Due to chemical constraints the longer synthesis way, i.e., enzymatic acylation of amino aldehyde semicarbazones, was found to be more efficient leading to higher overall product yields. Neat organic solvent systems were also used for the coupling of Z-Arg-OMe with various long-chain alkylamines (Scheme 15).[312] Using immobilized papain as the biocatalyst and acetonitrile with an aqueous buffer content ranging from 0 to 1% (v/v) as the solvent, product yields between 81 and 89% could be obtained. Under the best conditions, the syntheses were scaled up to the

Scheme 13. Synthesis of Z-Phe-Ag-OEt (Ag, allylglycine) from Z-Phe-OMe and L,D-Ag-OEt by Chymotrypsin Using a Biphasic Reaction System[a]



[a] According to ref 309 with permission from Elsevier Science. Copyright 1995.

Scheme 14. General Scheme of Semi-Enzymatic Peptide Aldehyde Synthesis[a]



[a] According to ref 311 with permission from Elsevier Science. Copyright 1999. Sem, semicarbazone; Pheol, phenylalaninol; Pheal, phenylalaninal.

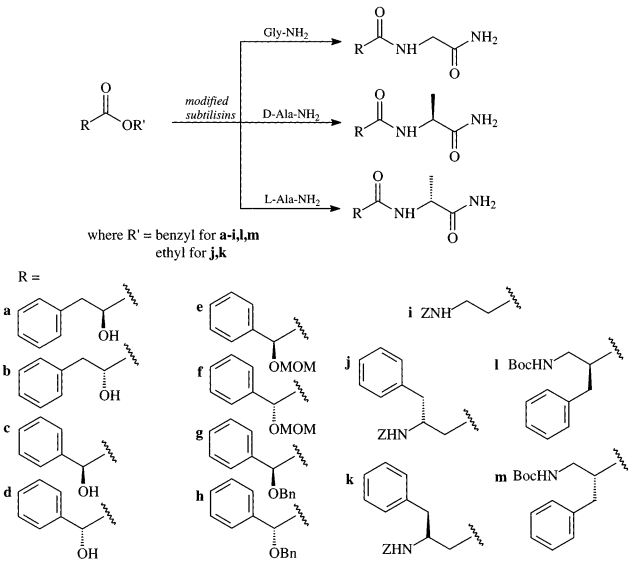
Scheme 15. General Scheme of the Acylation of Fatty Amines with Z-Arg-OMe Catalyzed by Immobilized Papain in Neat Organic Media[a]

[a] According to ref 312 with permission from John Wiley & Sons Inc. Copyright 1999. n = 7, 9, 11, 13, 15.

preparation of gram quantities of final product. The overall yields, which include enzymatic reaction, Z-group deprotection, and purification, varied from 53 to 77% of pure product. With similar yields, bis-arginine analogues containing various α,ω-diaminoalkanes as the linker between the two arginine moieties has been synthesized.[313] A two-step reaction strategy, in which the first arginine was found to react with the diamine spountaenously while the coupling of the second has been mediated by papain in a solid-to-solid system, gave the highest yields. A comprehensive study on the flexibility of subtilisin to acylate noncoded amino acid moieties in aqueous DMF mixtures has been published by the Wong group.[314] The researchers used a series of noncoded amino acids, amino acid precursors, and peptide fragments containing the statine-type isostere, peptide bond isosteres, and peptide mimetics as the acyl donor. From the results obtained, together with those found in previous studies, useful rules for each enzyme subsite between S_4 and S_3' regarding their preferences were concluded. As expected, the most restricted flexibilities were found for the S_1 and S_1' subsites of the enzyme. The specificity of the latter limits the choice of nucleophilic amines to those closely related to the structure of the preferred glycine. Although often applied, the acylation of noncoded amino acid moieties does not inevitably need to be performed in reaction mixtures with a high content of organic solvent. Recently, we could show that a large number of noncoded amino acid derivates and even non-amino acid-derived amines can be efficiently acylated in aqueous media using clostripain as the catalyst.[223] In fact, the enzyme is capable of acylating a broad variety of nucleophiles including aliphatic noncyclic and cyclic amines as well as amino alcohols, non-α-amino carboxylic acids, symmetric, and asymmetric diamines. Moreover, for most derivatives acylation rates could be observed higher than those found for specific acyl acceptors derived from coded amino acids indicating high efficient interactions of clostripain with these unnatural substrates. Interestingly, despite the efficient formation of the peptide isosteres, all synthesis reactions occur practically irreversible without any secondary hydrolysis of the product formed.

In contrast to the more relaxed specificity of some proteases toward the acyl acceptor, the specificity toward the donor component is usually more restricted, generally limiting the spectrum of accepted acyl donors to those closely related to preferred natural substrates. Broadening the limited enzyme specificity toward the donor component, however, can be achieved by medium, enzyme, and substrate engineering, as noted earlier (cf. chapter III). For example, Krix et al. used a solid-to-solid conversion approach to incorporate the Z-protected noncoded amino acids α-aminobutyric acid, homophenylalanine, phenylglycine, and neopentylglycine in several dipeptides.[310] Using thermolysin as the biocatalyst and the equilibrium synthesis approach, excellent yields ranging from 89 to 95% could be obtained. Another example demonstrated the coupling of allylglycine in P_1 position with phenylalanine amide

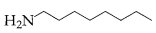
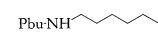
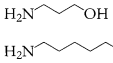
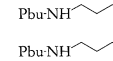
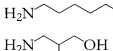
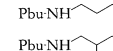
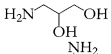
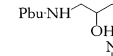
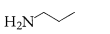
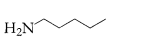
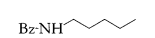
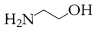
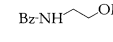
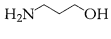
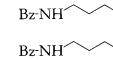
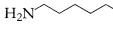
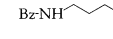
Scheme 16. Coupling of Glycine Amide and L-/D-alanine Amide with a Variety of Noncoded Carboxylic Acid Esters in 50% Aqueous DMF (v/v) Catalyzed by Chemically Active-Site Modified Subtilisin S166C Variants[a]



[a] According to ref 241 with permission from Elsevier Science. Copyright 2001.

mediated by various proteases, such as Nagarse, Pronase E, and the proteases from *A. oryzae* and *A. sojae*.[309] Starting from a racemic allylglycine derivative, yields around 20% of the L,L-dipeptide have been obtained using either an aqueous−organic solvent mixture (70% acetonitrile (v/v)) or a biphasic reaction system. The acceptance of phosphorylated tyrosine moieties at P_1 position by subtilisin has been investigated by Wong and co-workers.[314] Due to the preference of the enzyme for hydrophobic amino acid residues in this position, the phosphate moiety was used in its protected fashion. Reactions in about 60% DMF (v/v) with glycine amide as the nucleophile were successful in instances in which the protecting group of the phosphate was small, e.g., methyl or ethyl, while bulky protecting groups hinder the reaction. The utility of enzyme engineering for broadening the substrate acceptance of subtilisin was investigated by the laboratory of Jones.[242] As already noted earlier (cf. chapter III), the authors used a combination of site-directed mutagenesis and chemical modification to modulate the activity and specificity of subtilisin. By linking of aromatic or aliphatic low-molecular weight ligands to the artificial Cys^{166} of subtilisin S166C, a variety of noncoded carboxylic acids, including β-alanine and β-amino homologues of phenylalanine could be coupled with Gly-NH_2 and L-/D-Ala-NH_2 (Scheme 16). Despite the distinct structures of the active-site ligands, a closely related specificity was found for the corresponding enzyme species. Accordingly, the highest yields (between 27 and 79%) were obtained for α-benzylcarboxylate donors **a**, **b**, **l**, **m** consistent with the natural P_1 preference of the parent enzyme for phenylalanine. On the contrary, no reaction occurred with the acyl donors **c**, **d**, **j**, and **k**. Although significantly broadened over the wild type enzyme, the stereochemical preference of the modified enzymes typically favors the configuration

Table 10. Clostripain-Catalyzed Coupling of Non-Amino Acid-Derived Carboxyl and Amino Components[a]

acyl donor	acyl acceptor	amide product	yield (%)
Pbu-OGp			81
Pbu-OGp			80
Pbu-OGp			53
Pbu-OGp			65
Pbu-OGp			78
Pbu-OGp			70
Pbu-OGp			92
Pbu-OGp			95
Pbu-OGp			n. s.
Bz-OGp			82
Bz-OGp			76
Bz-OGp			56
Bz-OGp			57
Bz-OGp			84
Bz-OGp			70
Bz-OGp			82
Bz-OGp			94
Bz-OGp			n. s.

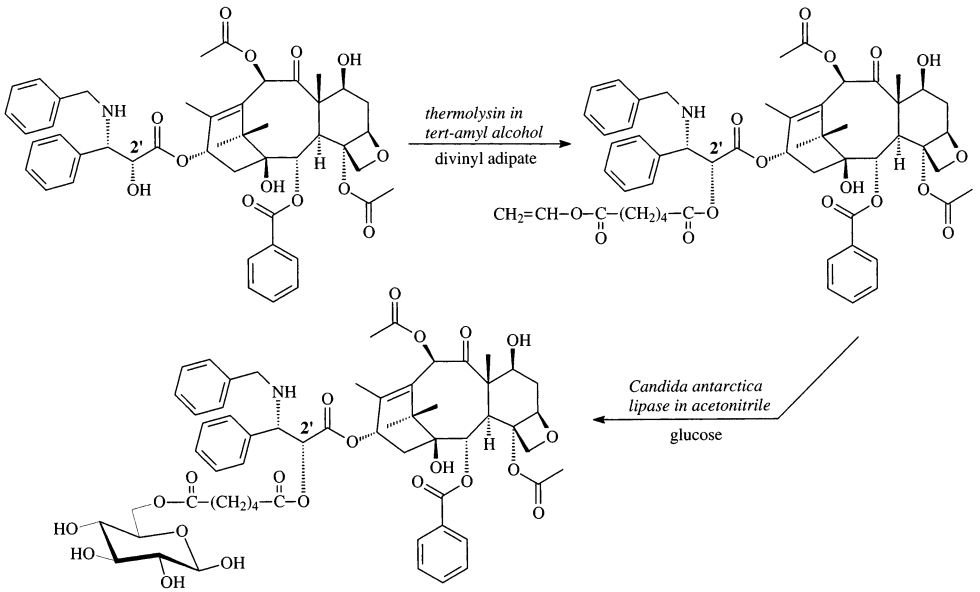
[a] According to ref 223. Maximum product yields after complete donor ester consumption are given. Conditions: 0.2 M HEPES-buffer (pH 8.0), 0.1 M NaCl, 0.01 M $CaCl_2$, 5% DMF, 25 °C, [acyl donor] = 2 mM, [acyl acceptor] = 12 mM. n. s., no synthesis.

of the α-benzyl stereocenter in these donors that is homochiral with that of L-phenylalanine. In addition to the broadened P_1 specificity, a more relaxed P_1' specificity was found for the modified subtilisin variants allowing the construction of all four possible stereoisomers of both **a**,**b** and **l**,**m** with L-/D-Ala-NH_2. Excellent tools for the coupling of a wide variety of carboxylic acids are substrate mimetics, as already mentioned earlier (cf. chapter III). More efficient than other methods, the use of substrate mimetics circumvents the specificity problem enabling proteases to react not only with nonspecific coded amino acids or closely related derivatives, but also with non-amino acid-derived acyl donors. Examples for this remarkable activity to the synthesis of peptide isosteres are already mentioned within chapter III. Besides the formation of peptide isosteres, the combination of the substrate mimetics strategy with the broad tolerance of clostripain toward the acyl acceptor also allows for the formation of completely nonpeptidic carboxylic acid amides.[223] Selected examples of this interesting approach are listed in Table 10. Importantly, the reactions proceed with a high catalytic efficiency and chemoselectivity as well. From the synthetic point of view, these findings show that the substrate mimetics strategy combined with the use of proteases possessing a broad specificity toward the amino component represents a powerful approach to the coupling of both non-amino acid-derived carboxyl and amino components. Thus, this approach opens up a new field of synthetic applications of these enzymes completely outside of peptide synthesis. This combines efficient and selective organic amid bond formations with the possibility of using extraordinarily mild reaction conditions.

C. Esterification/Transesterification

Esterification and transesterification, although leading by definition to identical synthesis products, i.e., carboxylic acid esters, proceed via completely different catalytic mechanisms. While for the latter an active-site nucleophile such as Ser or Cys is mechanistically essential,[315] esterification of free carboxylate moieties can be achieved, in principle, with all proteases by using the equilibrium-controlled synthesis approach (cf. chaper II). Neither for esterification nor transesterification, however, are proteases the most popular choice. The main reason for this is the high substrate specificity of most proteases that usually limits their synthetic utility to the coupling of acyl acceptors closely related to preferred amino acid residues. In this context, the use of esterases and particularly of lipases is generally more attractive.[54,316] Due to the broader substrate specificities of those enzymes, a wider structural diversity of carboxylic acid and alcohol moieties undergo the esterase- and lipase-catalyzed esterification and transesterification. Hence, it is not surprising that proteases have only found limited application in comparison to lipases and esterases. There are mainly secondary factors such as stability or unique selectivity which, however, make proteases attractive alternatives and, in a number of instances, even the better enzymes. Protease-catalyzed esterification and transesterification are mainly employed to resolve carboxylic acids and, in some cases, racemic alcohols and to stereoselectively acylate prochiral and meso diols. Furthermore, the selective acylation of carbohydrates or related compounds is a major field of application in this context. A selection of recent examples for these will be given in the following two sections. In addition, a number of papers are published in which the resolution of racemates was not the main focus. Some of them will be described and commented on here. An interesting example in this context was recently published by Khmelnitsky et al.[317] The authors report on a two-step enzymatic acylation of paclitaxel via selective transesterification to improve the poor water solubility of this powerful antimitotic agent (Scheme 17). In the first step, paclitaxel was thermolysin-catalyzed acylated at its C-2′ hydroxyl moiety using the bifunctional divinyl adipate as the acylating compound. In the second step, a lipase was used to selectively glycosylate the remaining activated carboxylate functionality of the adipate moiety. The capability of thermolysin, which is a zinc-containing protease without an active-site Ser or Cys, to catalyze this transesterification reaction contradicts to the statement given above. Unfortunately, this unusual finding was not further discussed by the authors. In another report, Clapes and co-workers applied the esterification and transesterification approach to the preparation of N^{α}-protected amino acid glyceryl esters starting from respective N^{α}-protected amino acid methyl or ethyl esters and nonesterified analogues, respectively.[318] From the eight proteases

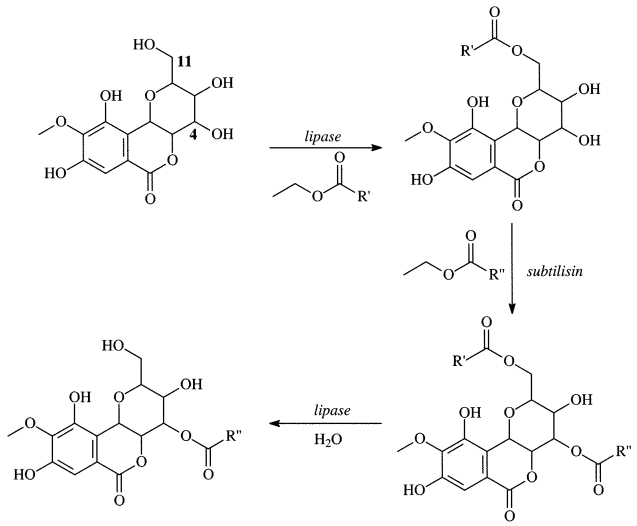
Scheme 17. Two-Step Enzymatic Modification of Paclitaxel via Transesterification[a]



[a] According to ref 317 with permission from the American Chemical Society. Copyright 1997.

and lipases tested, subtilisin was found to be the most efficient biocatalyst for the transesterification-based glyceryl ester synthesis. Yields of about 70% could be obtained in a reaction system containing 90% glycerol and 10% water (v/v). In contrast, the esterification activity under equilibrium-controlled conditions was marginal for this enzyme, whereas papain was as active as subtilisin in the esterification approach under these conditions. In contrast, a high esterification activity of subtilisin in 1,3-propanediol and 1,4-butanediol has been reported.[319] Importantly, the esterification reactions were regiospecific at the C-terminal C^{α}-carboxylate of Boc-protected amino acids and even of peptides up to a length of 21 amino acid residues. No hints to undesired peptide cleavages were found under these conditions. The peptide esters obtained by esterification were subsequently used as acyl donor components for papain- and subtiligase-catalyzed kinetically controlled peptide ligation. Another recent article reports on the use of the transesterification approach to produce a solution-phase combinatorial library of 167 distinct, selectively acylated derivatives of the polyhydroxylated flavonoid bergenin on a robotic workstation in a 96-well plate format.[320] Structural variety was achieved by subtilisin that acylates bergenin selectively at positions 4 and 11. Additionally, by exploiting different lipases, up to 600 derivatives of 4,11-mono- and diacylated bergenin could be automatically produced (Scheme 18).

Scheme 18. Synthetic Strategy for Production of Acylated Bergenin Derivatives by Three-Step Regioselective Enzymatic Acylation/Hydrolysis[a]

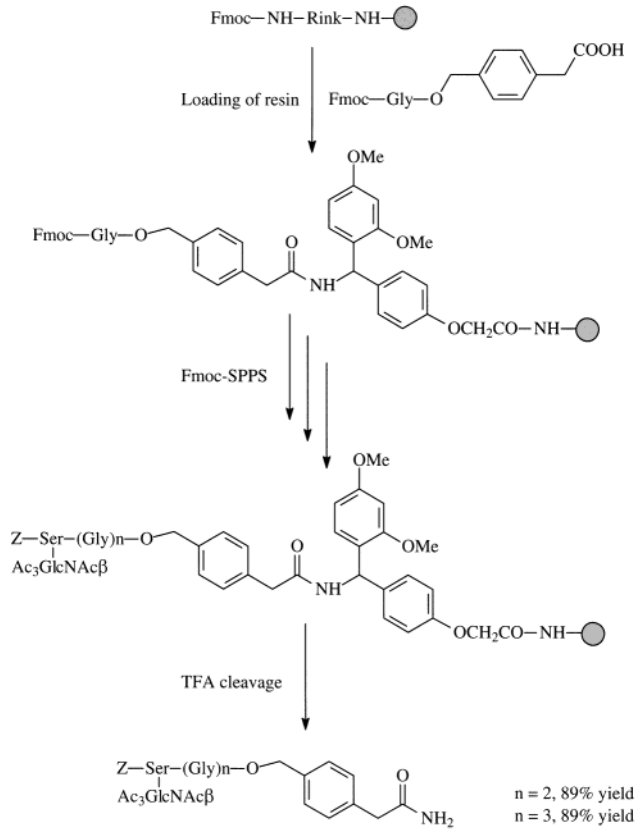


[a] According to ref 320 with permission from Elsevier Science. Copyright 1998.

D. Synthesis of Glycoconjugates

Synthesis of glycoconjugates by proteases can be achieved by two distinct ways: (i) ligation of glycopeptide and peptide building blocks by linking them through a new peptide bond and (ii) direct acylation of carbohydrates. In both cases, subtilisin has been a popular choice, but is not the only useful enzyme in this regard. The former way was mainly pioneered by Wong's research group. The first paper in this field appeared about one decade ago from the same laboratory.[321] In this comprehensive study, the researchers investigated the effect of the position of glycosylation within the donor and acceptor peptide on the ligase activity of subtilisin and two of its variants. It was found that with exception of the P_1 and P_1' position O- and N-glycosylation with monomeric as well as dimeric carbohydrates was tolerated by the enzymes. In selected cases, the synthesis products were further elongated at both the peptide backbone and the carbohydrate part by subtilisin and glycosyltransferases, respectively. A very similar approach even enabled the synthesis of glycosylated RNase B.[322] In the first step of this synthesis, a homogeneous variant of RNase B possessing only a single *N*-acetylglucosamine has been synthesized by treating the native, heterogeneously glycosylated

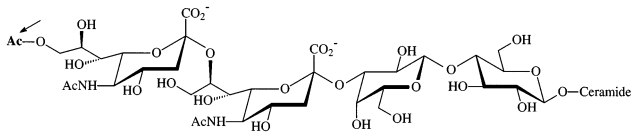
Scheme 19. Solid-Phase Synthesis of Glycopeptide 2-(1′-(hydroxymethyl)Phen-4′-yl)-acetamide (PAM) Esters[a]



[a] According to ref 324 with permission from the American Chemical Society. Copyright 1998.

enzyme with endoglucosidase H. Glycosyltransferases were then used to build a unique oligosaccharide moiety. Cleavage and religation of glycosylated RNase B by subtilisin, as originally described for its nonglycosylated counterpart RNase A,[323] finally opens up the door to manipulations of the protein part. Higher complexity of the synthesis products has been achieved also by combination of the enzymatic approach with solid-phase peptide synthesis methods.[324] For this purpose, a Fmoc-based solid-phase synthesis approach to the preparation of base- and acid-stable glycopeptide 2-(1′-(hydroxymethyl)phen-4′-yl)-acetamide (PAM) esters was established (Scheme 19). The resultant esters were subsequently used as the acyl donors in subtilisin-catalyzed model peptide bond formation. The utility of this methodology was further demonstrated by the subtilisin-mediated fragment condensation to give a 15-residue-long glycosylated peptide amide.

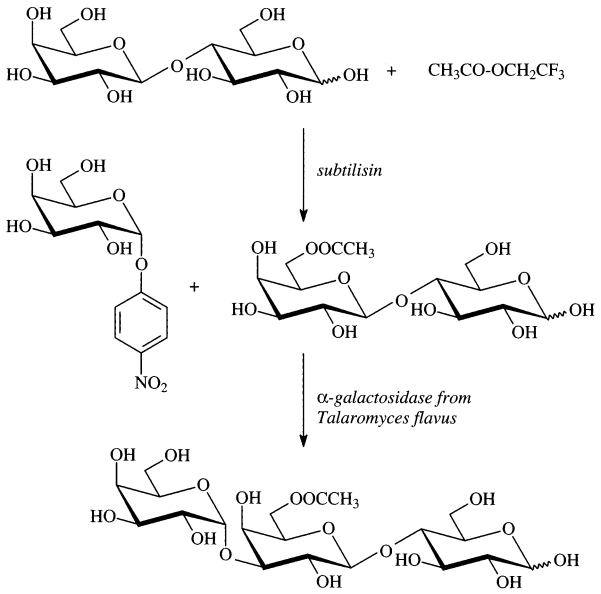
The direct acylation of carbohydrates represents a particular challenge due to the multiple hydroxyl groups of those compounds. Further difficulties result from the fact that proteases are not designed by evolution to recognize these biomolecules. Hence, additional manipulations on the reaction conditions are essential to achieve carbohydrate acylation. In general, hydrolase-mediated acylations of sugars are performed via an enzymatic transesterification of the appropriate acyl ester component. To circumvent the difficulties mentioned above, the process requires organic solvents as the reaction medium because in aqueous solutions water will replace sugar as a nucleophile, thus leading to hydrolysis instead of transesterification. However, sugars are reasonably soluble in only a few, very hydrophilic organic solvents such as pyridine and DMF. Furthermore, most enzymes in particular lipases, which would be predestinated for catalyzing transesterification reactions, are catalytically inactive in these solvents. To overcome this problem, Klibanow's group initiated the use of subtilisin as a potential catalyst due to its higher stability in the two organic solvents.[325] To compensate for the originally low rates of subtilisin-catalyzed reactions in neat DMF and pyridine (cf. chapter III), the researchers used activated esters, such as 2-chloroethanol or 2,2,2-trichloroethanol. By using this approach an enzyme efficiency remained that was sufficient to acylate mono-, di-, and oligosaccharides, as well as nucleosides and related large molecules in gram quantities. Interestingly, from the 10 carbohydrate compounds used, seven were exclusively monoacylated at their primary hydroxyl moieties with yields higher than 95%. In the case of the three remaining compounds the acylation at the primary hydroxyl was still the main reaction, but it was accompanied by some side acylations at other hydroxyl groups. Besides the optimization of subtilisin for carbohydrate acylations in DMF[228c] and the screening of other suitable proteases or protease preparations, later work has been mainly focused on the synthetic application of this approach. For example, Takayama et al. used the subtilisin in DMF approach to the synthesis of the disialoganglioside 9-*O*-acetyl GD3 (**13**) by regioselective acetylation of GD3.[326] In another work, Riva et al. have been studied the acylation of various di- and oligosaccharides containing a D-fructose moiety, such as maltulose, palatinose, turanose, or stachyose, using the activated ester trifluoroethyl butanoate as the acyl donor.[327]



(**13**)

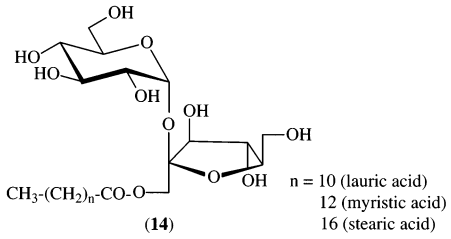
Applying a similar reaction system and subtilisin as the catalyst, all carbohydrates were converted into the corresponding monoesters. In all instances, a strong preference of the enzyme toward acylation of the primary C-1 hydroxyl of the fructose moiety has been found. Weignerova et al. used the high regioselectivity of subtilisin to acylate the 6′-*O*-position of lactose.[328] The partially protected lactose derivative was subsequently used as the acceptor in a transglycosylation reaction to achieve the synthesis of *iso*-globotriose without the formation of undesired 1→6 byproducts that hinders the use of free lactose as the reactant (Scheme 20). Another work continues on the protease-catalyzed regioselective synthesis of sugar-based surfactants originally described by Adelhorst et al.[329] This describes the synthesis of 1′-*O*-lauryl sucrose, 1′-*O*-myristyl sucrose, and 1′-*O*-stearyl su-

Scheme 20. Synthesis of *iso*-Globotriose (Galα(1→3)Galβ(1→4)Glc)[a]



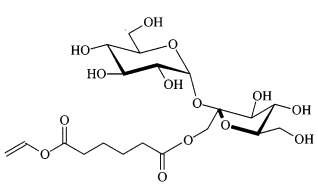
[a] According to ref 328 with permission from Elsevier Science. Copyright 1999.

crose (**14**) starting from sucrose and the appropriate fatty acid esters.[330] While the commonly used trichlo-

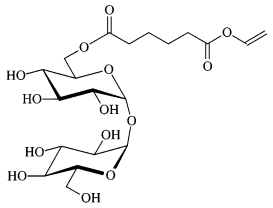


(**14**)

roethyl esters gave only low conversion rates, the corresponding vinyl esters were found to be markedly more reactive resulting in yields between 80 and 90%. The higher reactivity of vinyl esters, which were originally developed for chemical peptide synthesis,[331] has been already found in earlier studies and can be explained by the irreversible tautomerization of vinyl alcohol to unreactive aldehyde which drives the reaction to completion.[332] Sugar-based surfactants with slightly different architecture were also synthesized by Boyat et al.[333] Instead of coupling of the carbohydrate and fatty part directly, a spacer containing a dicarboxylic acid and an amino acid was incorporated between the sugar and fatty alcohol moieties by subtilisin catalysis. In another work, Kitagawa et al.[334] synthesized 5′-*O*-vinyladipoylthymidine (**15**) by transesterification of divinyladipate in DMF using an alkaline protease from *Streptomyces* sp. which was originally found by screening a series of enzymes that are active in DMF.[335] Subsequently, the vinyl thymidine ester was chemically polymerized to give a polymer having a molecular mass of 24 000 Da. The acylation of dimeric carbohydrates, such as sucrose and trehalose, with a dicarboxylic acid was investigated by Dordick and co-workers using subtilisin-like proteases from various sources.[336] Utilizing the best enzyme and divinyladipate as the acyl donor, the monoacylated glycoconjugates sucrose 1′-*O*-vinyladipate (**16**) and trehalose 6-*O*-vinyladipate (**17**) were found to be the major synthesis products (note that trehalose 6-*O*-vinyladipate is identical to trehalose 6′-*O*-vinyladipate, as the disaccharide is symmetrical).

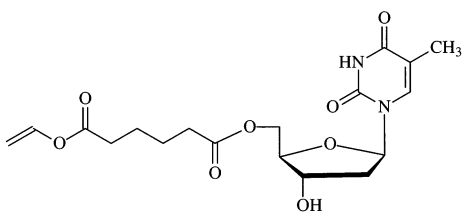


(**16**)



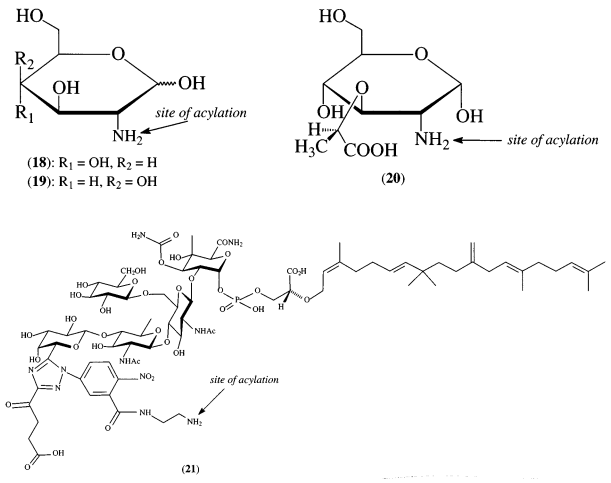
(**17**)

Similar results have been reported for the acylation of the two disaccharides using either methacrylates and laurates[337] or various N^{α}-protected amino acid esters as the acyl donor.[338] Boyer et al. continued on investigations using N^{α}-protected amino acid esters derived from phenylalanine, aspartic acid, and glutamic acid as the acyl donor and 16 monomeric carbohydrate derivatives as the acceptor components.[339] With exception of thioglycosides, which were used for the first time in subtilisin-catalyzed reactions, the high regioselectivity of the enzyme for the primary hydroxyl moieties has been verified. Unfortunately, but consistent with the well-known specificity of subtilisin, neither the diesters of aspartic acid nor glutamic acid were accepted as acyl donors. Similar reactions with CLEC-thermolysin also failed to accept the two donor components and again only the phenylalanine ester was accepted. The use of papain as the catalyst slightly broadens the range of accepted substrates to alanine, as indicated by the successful coupling of Z-protected alanine with sorbitol.[340] On the contrary, the chemoselective synthesis of N-linked neo-peptidoglycans can be achieved with a remarkably broad variety of peptide sequences.[225] The hallmark of this approach is the use of an ordinary peptidase as the biocatalyst, i.e., the cysteine peptidase clostripain from *Clostridium histolyticum*, combined with a novel type of substrate mimetics used as the amino acid and peptide precursors (cf. chapter III). Similar to the classical linear substrate mimetics, the novel type peptide donors bear a site-specific ester leaving group, i.e., the 4-guanidinophenyl ester moiety (OGp), that mediates the acceptance of nonspecific acyl residues by the original highly Arg-specific protease. However, to direct the enzyme's intrinsic synthesis activity to the side-chain of Asp and Glu, the specific OGp group is linked to the ω-carboxylate of the two amino acids instead of being at the C-terminus of the peptide. This different architecture was found to shift



(**15**)

the synthesis activity of the biocatalyst from the peptide's C^{α}-carboxylate toward that of Asp and Glu side-chains finally resulting in the synthesis of a broad spectrum of N-linked carbohydrate-peptide conjugates. In fact, the approach allowed for chemoselective coupling of the esterified ω-carboxylate moieties of Z-Glu(OGp)-OH, Z-Asp(OGp)-OH, Z-Asp(OGp)−Tyr−Gly−Gly−Phe−Leu-OH, and the C-terminus of Boc-Phe−Gly−Gly-OGp with both simple monomeric and highly complex carbohydrate derivatives, such as D-glucosamine (**18**), D-galactosamine (**19**), muramic acid (**20**), and moenomycin A (**21**), under mild aqueous reaction conditions with yields ranging between 24 and 73%. Presently, there is no other enzyme system that shows a similar synthetic flexibility toward both the peptide and the carbohydrate part making this approach a powerful and rather general one for the synthesis of N-linked neopeptidoglycans and amino acid-carbohydrate building blocks.



(**18**): R_1 = OH, R_2 = H
(**19**): R_1 = H, R_2 = OH
(**20**)
(**21**)

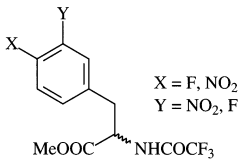
E. Kinetic Resolution

Kinetic resolution is defined as a process in which one of the enantiomers ($R + S$) of a racemic mixture is more readily transformed into a product than its mirror image. From a kinetic point of view, resolution of enantiomers occurs when $k_R \neq k_S$ and when the reaction is stopped at some stage at or near 50% conversion. Quantification of the enantioselective performance of an enzyme is generally expressed as the (dimensionless) enantiomeric ratio E, which is considered as the ratio of the second-order rate constants k_{cat}/K_m for the two enantiomers. Equations have been developed that relate E to the value of the enantiomeric excess of the substrate (ee_S) or of the product (ee_P).[341] Alternatively, E can be more conveniently calculated from the degree of conversion and from the enantiomeric excesses of the products and the residual substrate.[342] In every case, the higher the value for E, the higher the enantiomeric excess of the product and of the residual substrate, whereas an E of 15 is usually considered as the lower limit for practical purposes. In contrast, enantiomeric ratios exceeding a value of 30 can be regarded as excellent.

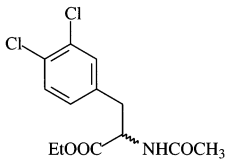
In general, resolution of enantiomers by protease-catalysis can be achieved by the whole spectrum of reactions that can be mediated by these enzymes, i.e., hydrolysis of carboxylic acid esters or amides, esterification, transesterification, and amide bond formation. However, the already noted restricted substrate specificity, which is equally important for all of these reactions, limits the synthetic utility of proteases to the resolution of racemic compounds closely related to preferred amino acid moieties while the resolution of non-amino acid-derived counterparts is the domain of esterases and lipases. Therefore, it is not surprising that the greater part of papers reporting on protease-mediated resolution of enantiomers is focused on amino acids and their structural homologues. On the contrary, only a limited number of applications have been reported on the resolution of compounds that one would not necessarily regard as protease substrates. A selection of recent examples will be presented in the following section. A more detailed overview including lipases and esterases can be found in several recent reviews.[343]

One of the most preferable protease-based approaches to the resolution of racemic amino acids represents the enantioselective hydrolysis of their esters. Due to their broader substrate specificity, subtilisin, chymotrypsin, and the protease from *A. oryzae* are the by far most popular proteases for this purpose. In parallel to the native enzyme specificity, the three proteases catalyze the hydrolysis of L-amino acid ester derivatives leaving the D-counterparts unaffected. Besides this classical finding, current research focuses on the use of this approach to the resolution of synthetic noncoded amino acid homologues. This reflects that for the supply of noncoded amino acids in quantity, the chemical synthesis of racemic forms followed by their optical resolution is still the preferable way, although a number of methods have been developed for the asymmetric synthesis of amino acids.[344] Recently, the resolution of a series of ring-substituted phenylalanine derivates has been published by several laboratories. For example, Vergne et al. demonstrated the successful resolution of nitro- and fluoro-substituted phenylalanine derivatives (**22**) by subtilisin-catalyzed hydrolysis of (*R,S*)-*N*-trifluoroacetyl-3(4)-fluoro-4(3)-nitro phenylalanine methyl esters.[345] Further related examples that exist in the scientific literature describe the resolution of halogenated phenylalanine derivatives such as **23**,[346] γ,δ-unsaturated phenylnorvaline homologues (**24**),[347] ring-size modified amino acids (**25**),[348] or α,β-disubstituted β-phenylalanine derivatives such as *threo*-methylphenidate (**26**)[349] catalyzed either by chymotrypsin or subtilisin. Similarly, the protease from *A. oryzae* was found to be highly useful for the resolution of a series of halogenated phenylalanine esters with and without *N*-terminal protection.[350] In cases where the enzyme enantioselectivities have been low, the use of ester moieties with longer alkyl chains and/or lowering the temperature markedly enhanced the optical purity of the products. In another report, Kapeller et al. used the same enzyme for the resolution of the isoxazoline derivative of 2-amino-4-hydroxy-4-(4-methyloxyphenyl)-3-methylbutanoic acid methyl ester (**27**) which represents a key intermediate in the

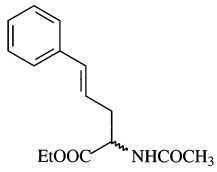
synthesis of the nucleoside antibiotic Nikkomycin B.[351] The resolution was performed in a biphasic



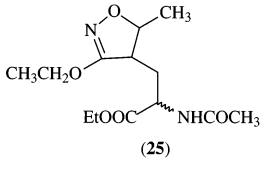
(22)



(23)



(24)



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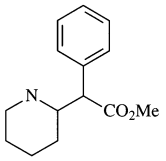
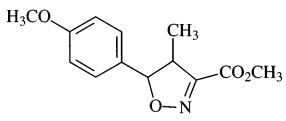


(26)

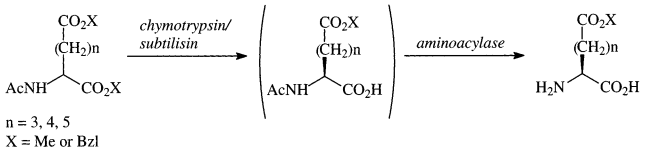


(27)

toluene/phosphate buffer system, whereas the enzyme specifically hydrolyzed the (*R*,*R*)-configured ester of racemic **27**. Racemates of noncoded amino acids with aliphatic side-chains can also be resolved by these enzymes. For example, racemic esters of norvaline, norleucine, or 2-aminobutanoic acid can be resolved by the *A. oryzae* enzyme in enantiomeric ratios *E* mostly higher than 30.[349] Optically pure D-*tert*-leucine was obtained by enantioselective hydrolysis of *N*-acetyl-D,L-*tert*-leucine chloroethyl ester using alcalase from *Bacillus licheniformis*—a crude protease mixture containing mainly subtilisin—and subsequent acidic saponification of the recovered ester.[352] A high enantiomeric excess has been also reported for the resolution of *Z*-γ,γ'-di-*tert*-butyl-D,L-carboxyglutamic acid methyl ester by using papain as the catalyst and aqueous−organic mixtures as the solvent.[353] Fully protected racemic α-aminoalkanedioic acid diesters can be resoluted with the help of chymotrypsin leading to the optically pure L-amino acid monoesters in yields of about 75% based on the corresponding L-amino acid diester in the racemic mixture.[354] Quantitative yields have been achieved by the combined action of chymotrypsin and subtilisin. Additional one-pot deacetylation using an L-specific aminoacylase improved the workup procedure and, hence, the yields of the desired enantiomers (Scheme 21).

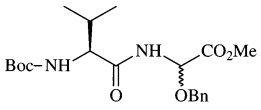
A later study has been shown that resolution of *Z*-D,L-2-aminosuberic acid methyl diester ($n = 5$, Scheme 21) can also be achieved by papain catalysis or, alternatively, by subtilisin alone using an organic solvent system with low water content.[355] Subtilisin can be also helpful in the synthesis of optically pure α-hydroxyglycine peptides.[356] Resolution of those peptides has been achieved during the synthesis process on the stage of the intermediate α-acetoxy-

Scheme 21. Tandem Enzymatic Resolution of Racemic α-Aminoalkanedioic Acid Dieser[a]



[a] According to ref 354 with permission from the Pharmaceutical Society of Japan. Copyright 1996.

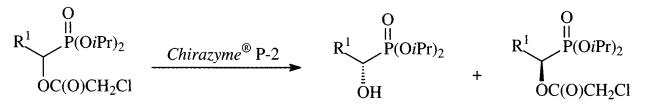
glycine peptide (**28**). After separation, both stereoisomers could be obtained in nearly quantitative yield



(28)

with a diastereomeric excess of >99%. Hydrogenolysis of separated **28** finally led to the desired optically pure α-hydroxyglycine peptide. In another recent work, the subtilisin-like protease Chirazyme P-2 was used for the chemoenzymatic synthesis of optically pure phosphonic acid analogues of L-leucine, L-isoleucine, L-methionine, and L-α-aminobutyric acid.[357] Resolution of the enantiomers was performed on the stage of the α-chloroacetoxyphosphonate intermediates with *ee*'s of the recovered products between 92 and 99% (Scheme 22). Transformation of the resolved

Scheme 22. Resolution of α-Chloroacetoxyphosphonates by Chirazyme P-2[a]

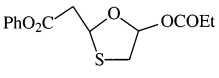


R^1	yield (%)	*ee* (%)
*i*Pr	40	92
*i*Bu	39	99
$MeS(CH_2)_2$	27	98
(2 *S*)-*s*Bu	35	98

[a] According to ref 357 with permission from Elsevier Science. Copyright 1999.

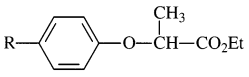
α-hydroxyphosphonates into the corresponding aminophosphonic acids proceeds with inversion of configuration involving the replacement of hydroxyls with azide under Mitsunobu conditions and subsequent reduction of the azides to the amines.

As mentioned above, examples for the resolution of compounds that show a lower degree of structural relationship to preferred amino acid moieties of the enzyme are more rarely. For those compounds not only low reaction rates are usually found, but also a reduced enantioselectivity. For example, the resolution of the racemic oxathiolane benzoate derivative **29** by subtilisin only proceeds with an *ee* of 15%, while trypsin showed no enantioselectivity at all.[358]



(29)

Although somewhat improved, the enantiomeric ratios *E* of subtilisin for the resolution of 2-(4-substituted phenoxy)propionates (**30**) were found to be only

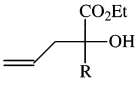


R = H, CH_3, CH_3CH_2, $CH_3(CH_2)_2$, $CH_3(CH_2)_3$, $CH_3(CH_2)_5$

(**30**)

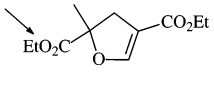
in the range of 2.2−6.3 corresponding to *ee*'s between 29.9 and 61.7%.[359] Additionally, a number of further examples are reported in the scientific literature, while the greatest part of such negative results certainly vanished in the desk drawers of the researches. In some instances, medium, substrate, and enzyme engineering may enhance the enantioselectivity of proteases toward those nonspecific substrates (cf. chapter III). For the resolution of **30**, for example, the addition of distinct volumes of DMSO to the reaction mixture increases the enantioselectivity of subtilisin leading to *E* values ranging from 7.6 to 23. The effect of DMSO was postulated to be directly related to conformational changes of the biocatalyst which increase the flexibility of the protein and finally lead to the improvements of enantioselectivity observed. Time-consuming optimization of the solvent composition represents the general drawback of this approach. Presently, the screening of large enzyme libraries is the more preferable methodology while medium engineering is mainly used for fine-tuning of the selected biocatalyst.

Sterically hindered carboxyl esters bearing a fully substituted quaternary carbon adjacent to the ester moiety, i.e., esters of α,α-disubstituted carboxylates and of *tert*-alcohols, are usually not accepted as substrates of hydrolases, also when they are structurally related to specific amino acid moieties.[343b] Exceptions to this rule have been reported for compounds in which at least one of the α-substituents exerts electron-withdrawing effects, e.g., through a heteroatom (O, N), which obviously makes these sterically demanding esters better accepted. For example, Feichter et al. have been reported on the resolution of compounds **31a** and **31b** by *A. oryzae*

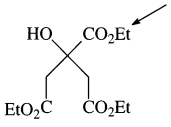


(**31a**) R = Ph
(**31b**) R = CH3

protease in an enantiomeric ratio *E* of 26 (*ee* = 88%).[360] The same enzyme also accepts cyclic derivatives of α,α-disubstituted carboxylic acid esters such as the α-amino-γ-hydroxycarboxylic acid **32**. Interestingly, hydrolysis of this compound occurred only at

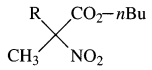


(**32**)



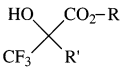
(**33**)

the more sterically hindered α,α-disubstituted carboxyl ester group with good enantioselectivity (*E* = 35).[361] A similar finding was reported for the hydrolysis of the triethyl citrate ester **33**.[362] Both chymotrypsin and subtilisin regiospecifically hydrolyzed the carboxyl ester moiety directly linked to quaternary (but symmetric) carbon atom remaining the two others unaffected. The ability of proteases to differentiate between distinct moieties of similar reactivity is presently an attractive approach to the enzymatic desymmetrization of prochiral and meso compounds and has been recently reviewed in detail.[363] α-Substituted α-nitropropanoate esters such as **34**, which bear an electronic-withdrawing "hidden" amino group and constitute therefore versatile starting materials for the synthesis of the corresponding α-amino acids, also act as substrates and can be resolved by chymotrypsin.[364] A similar behavior was recently found for the CF_3 moiety of α-trifluoromethyl-α-hydroxycarboxylic acid esters such as **35a**−**d**.[365] A screening of a series of approximately 30



R = $CH_2CH{=}CH_2$, Ph, 3-indolyl, $(CH_2)_2COCH_3$

(**34**)

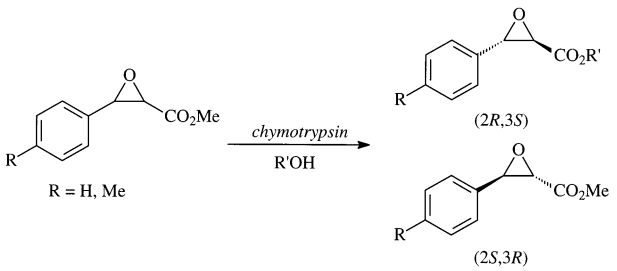


(**35a**) R = R' = Me
(**35b**) R = Bn, R' = Me
(**35c**) R = Et, R' = Me
(**35d**) R = Me, R' = Et

hydrolases revealed subtilisin as the most promising lead for these reactions. Generally, the selectivities obtained during these studies were rather low (*E* = 7). However, at high conversion, the remaining untouched esters of **35a** and **35b** could be separated in *ee*'s higher than 95 and 90%, respectively. Additionally it should be noted that in search for novel proteases, which tolerates α,α-disubstitution, two remarkable amino acid amidases from *Mycobacterium neoaurum* and *Ochrobacterium anthropi* could be identified from an extensive screening program by using α,α-disubstituted glycine amides as substrates.[366]

Compared to the resolution of racemic carboxylic acids by hydrolysis of their esters, protease-mediated resolution by corresponding transesterification reactions is by far less popular. Three reasons mainly account to this fact: (i) transesterification essentially needs to be performed in neat organic solvents; (ii) transesterifications are reactions that frequently proceed in an uncomplete manner; and (iii) the products of transesterifications have similar physicochemical properties which may complicate purification especially when chromatographic techniques are used. Similarly, resolution of carboxylic acid esters by aminolysis typically suffers from an uncomplete conversion mainly due to hydrolytic side reactions that lowers the yield and increases the product spectrum. Furthermore, the newly formed amide bond cannot be cleaved by simple saponification and, thus, permanently modifies the synthesis product. These drawbacks are reflected by the limited number of recent papers that report on transesterification and aminolysis approaches to the resolution of racemic carboxylic acids. One of these rare examples have been published by Gentile et al.[367] The authors used

Scheme 23. Kinetic Resolution of *trans*-Phenylglycidic Methyl Esters by Chymotrypsin-Catalyzed Transesterification[a]



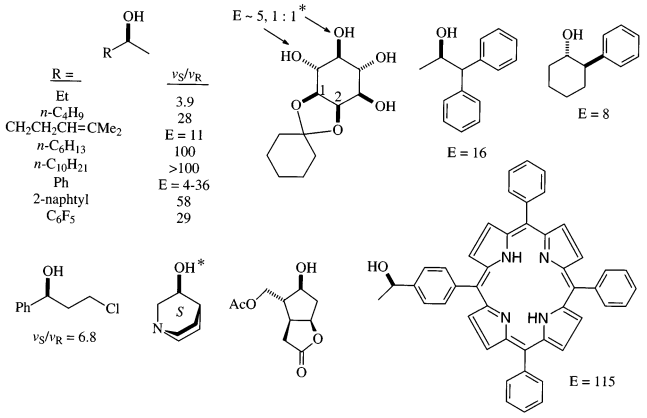
[a] According to ref 367 with permission of the American Chemical Society. Copyright 1992.

the transesterification methodology for the kinetic resolution of *trans*-phenylglycidic methyl esters, whose (2*R*,3*S*)-enantiomers are precursors of the calcium channel blocker drug diltiazem (Scheme 23). The enzymatic resolution has been achieved by chymotrypsin suspended in hexane/1-butanol. Further studies on transesterification-based resolutions are mainly focused on mechanistic backgrounds of this method or on approaches to improve the outcome of such reactions. For example, Broos et al.[368] studied the behavior of four different proteases, i.e., chymotrypsin, subtilisin, *A. oryzae* protease, and elastase, as the biocatalysts for the enantioselective transesterification of various *N*-acetyl-D,L-phenylalanine esters with 1-propanol in cyclohexane. This study revealed that the enantioselectivity of the reactions is not only affected by the nature of the enzyme, but also by the ester leaving group and the nature and amount of organic additives. From the results obtained, an empirical rule has been postulated after what addenda with a small molecular volume like, e.g., ethanol and acetonitrile, increase the rate for the L-enantiomer whereas alcohols with bulky alkyl groups such as *tert*-butyl alcohol and 2-methylbutan-2-ol enhance the activity of the ezymes toward the D-counterpart. From the same laboratory, a related work has been published that report on the influence of 18-crown-6 on the enantioselective performance of similar transesterification reactions.[369] Although the crown ether did not affect the enzyme's enantioselectivity directly, it enhanced the rate of the reaction and, hence, the degree of conversion. On the contrary, a direct effect of additives on the enantioselectivity was recently demonstrated for DMSO.[370] As already found for the resolution of **30** by means of ester hydrolysis, the addition of distinct volumes of DMSO to the reaction mixture also increased the enantioselectivity of subtilisin in corresponding transesterification reactions. Additional ESR spectroscopic studies have been verified that conformational changes of the biocatalyst caused by the organic additive could be the reason for the enhanced enantioselectivity.

Examples for the protease-catalyzed enantioselective aminolysis of racemic carboxylic acid esters are even more rarely than those mediated by transesterification. In fact, only three papers were found that have been published in the period of reviewing. Two of them focus on the enantioselective amidation (ammoniolysis) of racemic amino acid esters using either N^{α}-*Z*-[371] or N^{α}-Boc-[372] protected D,L-amino acid methyl esters. The resolutions were performed in *tert*-butyl alcohol saturated with ammonia utilizing six different proteases, all of them immobilized on Accurel EP100. For the two substrate series high enantioselectivities of the enzymes are reported, whereas the Boc-protected esters have been even resoluted with nearly absolute enantioselectivity. Unfortunately, these excellent enantioselectivities were generally accompanied by low conversion rates of less than 15%. The third article reports on the papain-mediated resolution of racemic *Z*-γ,γ'-di-*tert*-butyl-D,L-carboxyglutamic acid methyl ester via diastereoselective synthesis of various dipeptide amides and esters in organic low-water systems.[373] According to the acyl acceptor specificity of the enzyme, the highest yields of diastereomerically pure dipeptides have been optained for reactions with hydrophobic amino acid amides and esters as the acyl acceptors while hydrolysis of the L-configured carboxyglutamic acid ester was the only side reaction.

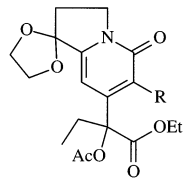
Besides the resolution of racemic carboxylic acids, hydrolases also affect resolution of chiral secondary alcohols. Due to the binding of the alcohol component at the less specific S′ subsites of the enzyme, esterification, and, more commonly, transesterification are the preferred approaches to achieve alcohol resolution. On the contrary, alcohol resolution by hydrolysis often displays lower enantioselectivities for the alcohol part due to the dominance of enzyme interactions to the carboxyl component over those between the enzyme and the alcohol moiety which are significantly weaker in most cases. Also, this low structural selectivity of the S′ subsites of proteases reduces the enantioselective ratio of esterification and transesterification reactions and usually makes protease-mediated alcohol resolution less stereoselective compared to that catalyzed by lipases and esterases. Hence, it is not surprising that the resolution of racemic alcohols is the domain of the latter enzymes, while proteases have found only limited application. Similarly, attempts to optimize or rationalize the two approaches have been originally performed with esterases or lipases, but are, in principle, equally important for proteases. In this context, strategies that make transesterifications irreversible have to be mentioned. This goal can be achieved with ester leaving groups that are poor nucleophiles and, hence, are unable to act as competitive reactants once they are released from the carboxyl component. Besides leaving groups derived from halogenated alcohols such as trichloro- and trifluoroethanol[374] or the use of oximes[375] and anhydrides,[376] especially vinyl esters[332] have gained much attention. As mentioned earlier, vinyl esters, once they are cleaved, underlie an irreversible tautomerization of the released vinyl alcohol to unreactive aldehyde which drives the reaction to completion. Presently, almost all papers that report on the protease-mediated resolution of racemic alcohols utilize subtilisin as the biocatalyst. This is in alignment with the high stability of this enzyme in organic solvents and its relatively broad substrate specificity, together with the ease of availability and low cost. Nevertheless, despite the strong focus on one single enzyme, its use as preparative

Scheme 24. Selection of Alcohols that Have Been Resoluted by Subtilisin[a]



[a] According to refs 377, 378, and refs therein. Resolution was performed either by esterification or transesterification. If possible, enantioselectivity is reported as the *E* values or the relative initial rates of reaction (v_S/v_R) toward the two enantiomers. Exceptions to the enzyme's selectivity toward the *S*-enantiomer of the alcohol are marked by an asterisk.

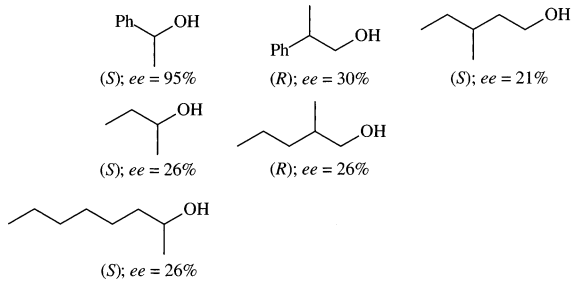
catalyst for the resolution of alcohols is still an exception. In fact, a great part of reactions performed so far focuses on mechanistic properties, but are nevertheless suitable to illustrate the general synthetic utility of this enzyme for the resolution of racemic alcohols. A selection of accepted alcohols, together with the corresponding enantioselective ratios for their resolution (as far as reported), have been recently summarized by Kazlauskas and Weissfloch[377] and Ema et al.[378] within their attempts to rationalize the *S*-enantiopreference of the enzyme toward secondary alcohols known from former studies (Scheme 24). Note that lipases and esterases react faster with the *R*-counterpart and, hence, show an opposite enantiopreference to subtilisin. First empirical rules that predict the enantiopreference of subtilisin stem from Fitzpatrick and Klibanov.[379] According to these, hydrolases discriminate between enantiomers primarily by the size of the substitutents linked to the chiral carbon atom. Generally, the more unbalanced the bulkiness of the two substituents attached to the stereocenter, the higher the enzyme's enantioselectivity. The opposite enantioselectivities of lipases/esterases and subtilisin have been related to their mirror image like catalytic machineries.[380] Kazlauskas and Weissfloch's proposal for the molecular basis of subtilisin's enantiopreference focuses on the protein fold. This fold both sets the absolute configuration of the catalytic machinery (Ser−His−Asp triad and the oxanion hole) and creates a restricted pocket for one substituent in the substrate. The proposal of Ema et al. confirmed the importance of the arrangement of the catalytic machinery and indicates that chiral discrimination of the enzyme originates from the transition state and not from the substrate binding step. Compared to the resolution of secondary alcohols, primary and tertiaery alcohols are less popular targets. The latter suffer from their sterical bulkiness which usually hinders the acceptance by the enzyme. The resolution of racemic tetrahydroindolizinyl butanoate derivatives (**36**) by enantioselective deacetylation is one of the very rare exceptions to this rule.[381] From the about a hundred commercially available hydrolases tested, *A. oryzae*



(**36**)

protease was found to exhibit the highest enantioselectivity. In fact, the *S*-configured *tert*-alcohols could be obtained in excellent *ee*'s of 98% and in moderate yields as the remaining substrates. The electrostatic activation of the ester bond by the adjacent carboxyl ester moiety of the alcohols has been postulated to be the reason for the acception of these sterically hindered alcohols. Unfortunately, no data have been reported for the corresponding esterification or the transesterification using deacetylated **36** as the nucleophile. Another work reports on the ability of thermitase, an alkaline serine protease from the thermophilic microorganism *Thermoactinomyces vulgaris*, for hydrolyzing *tert*-butyl esters of differently *N*-protected peptides.[382] Although *tert*-butyl alcohol is an achiral alcohol and, thus, no conclusions to the enantioselectivity of the reaction could be made, the high activity of the protease toward this ester makes thermitase an interesting biocatalyst being worth to be further investigated. Initial data for the resolution of primary alcohols have been recently reported by Jones and co-workers.[383] The authors compared the enantioselectivity of subtilisin toward β- and γ-branched primary alcohols with that already known for secondary alcohols in transesterification reactions. Their data are shown in Scheme 25 and illustrate a reduced enan-

Scheme 25. Resolution of Secondary and β- and γ-Branched Primary Alcohols by Subtilisin-Catalyzed Transesterification of *N*-Acetyl-L-phenylalanine Vinyl Ester[a]



[a] According to ref 383 with permission from Elsevier Science. Copyright 1998.

tioselectivity of the enzyme toward primary alcohols compared to that observed for secondary alcohols. This finding suggests that the enantiomeric discrimination of the enzyme decreases with the distance between the stereocenter and the reactive hydroxyl group—a finding which is in line with the well-known effect of spacers on the acceptance of racemic *tert*-alcohols by several hydrolases.[343b] Interestingly, a

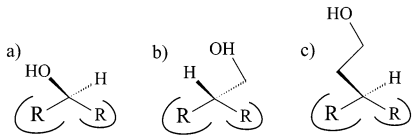
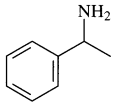


Figure 15. Model to predict and explain the specificity of subtilisin for (a) secondary alcohols, (b) β-branched primary alcohols, (c) γ-branched primary alcohols (according to ref 383 with permission from Elsevier Science. Copyright 1998). The size of 'R' denotes the relative size of the substituents.

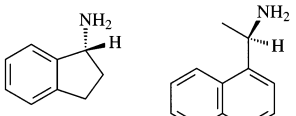
distinct enantioselectivity pattern of the enzyme towards the different alcohols became evident. While for the secondary alcohols the known *S*-preference has been verified, a reversal of selectivity favoring the *R*-enantiomer was apparent for β-branched primary alcohols. In the case of the single γ-branched derivative used, the selectivity reversed once more resulting again in an *S*-preference of the enzyme. Building on the model proposals of Klibanov[379a] and Kazlauskas,[384] the researchers rationalized their data by the models depicted in Figure 15.

Besides carboxylic acids and alcohols, amines represent the third important class of compounds that can be resolved by proteases. Due to their structural similarity and binding at the same enzyme subsite, the resolution of amines and alcohols occurs with similar enantiopreferences. Accordingly, proteases usually react faster with the *S*-enantiomer of amines in which the chiral carbon atom is directly adjacent to the reactive amine moiety. This classical finding, originally observed for the hydrolysis and the reverse of hydrolysis of peptides,[7a] has gained some industrial importance. For example, the multiton production of the artificial sweetener aspartame (L-aspartyl-L-phenylalanine methyl ester) starts from *Z*-L-Asp-OH and the racemic D,L-Phe-OMe as the reactants.[385] Due to the enantioselectivity of the preferred enzyme, i.e., thermolysin, only the L-enantiomer of the phenylalanine ester undergo the coupling reaction yielding to the optically pure L,L-dipeptide. Also, attention has been paid to the resolution of non-amino acid-derived amines. In this context, it should be mentioned that the first report on this subject has utilized a protease, i.e., subtilisin.[386] In this original work, kinetic resolution of various amines such as α-methylbenzylamine (**37**) was achieved by using an organic solvent system.



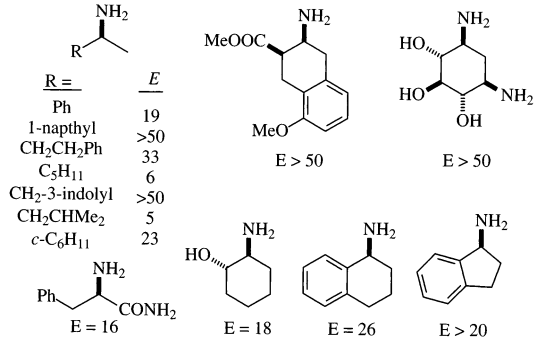
(**37**)

Later work has used subtilisin, for example, for the production of the pharmaceutically important intermediate (*R*)-1-aminoidan (**38**) and the chiral resolving agent (*R*)-1-(1-naphthyl)ethylamine (**39**).[387] By utiliz-



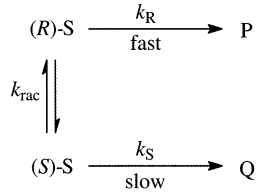
(**38**) (**39**)

Scheme 26. Selection of Amines that Have Been Resoluted by Subtilisin[a]



[a] According to ref 377, and refs therein. Resolution was performed by acylation with trifluoroethyl butyrate or alkoxycarbonylation with diallyl carbonate. The enatioselectivity is reported as the *E* value.

Scheme 27. Kinetics of "Dynamic Kinetic Resolution"[a]

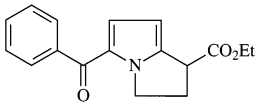


[a] The approach comprises a classic kinetic resolution and the in situ racemization of the starting material. (*R*)-S, (*S*)-S, substrate enantiomers; P, Q, product enantimers; k_R, k_S, individual rate constants; k_{rac}, racemization constant.

ing a continuous-flow reactor and immobilized subtilisin, the two compounds were resolved in a multigram- and kilogram-scale in *ee*'s > 98% and yields of 40 and 35%, respectively. A number of further examples have been found in the scientific literature and the amines used in these studies are summarized in Scheme 26. Interestingly, by comparing the enantiomeric ratios obtained for amine resolutions with those found for comparable alcohols (cf. Scheme 24), it becomes evident that the former usually occur with higher enantioselectivities. This behavior can be explained by taking into consideration the lower reactivity of amides whose hydrolysis need a more accurately binding to enzyme.

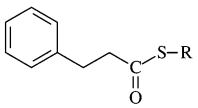
A general disadvantage of standard kinetic resolution procedures described so far is that a maximum 50% yield of the desired product enantiomer is obtained based on racemic starting material. To overcome this limitation, recovered starting material may in some cases be racemized and resubmitted to the resolution procedure. As a potentially more efficient approach, resolution processes have been coupled with continuous in situ racemization of the starting material as illustrated in Scheme 27. In principle, this permits quantitative conversion of racemic starting compounds into one enantiomer of the product in a single deracemization process. To indicate the nonstatic behavior of this process, the term "dynamic kinetic resolution" has been coined for this kind of resolution.[388] The kinetics of dynamic resolutions, which can be considered as a type of second order asymmetric transformations, have been recently reviewed.[389] Accordingly, the efficiency of the

dynamic resolution is highly influenced by the kinetic parameters of the parallel reactions and the racemization. To achieve efficient deracemization, several conditions must be fulfilled. The most important are (i) the racemization process (k_{rac}) has to be at least equal to, or faster than, the transformation of the faster reacting enantiomer (k_R). As this is not the case, the dynamic process gradually turns into a classic kinetic resolution process and ee_P is therefore depleted; and (ii) since the maximum obtainable ee_P in a dynamic resolution is equal to the ee_P at very low conversion in classic kinetic resolutions, the enzyme's enantioselectivity should be as high as possible with E values at around 20 being the lower limit. Of course, in situ racemization of the substrate inevitably represents the most challenging task of dynamic resolution. Generally, this process occurs by breaking and forming bonds and can be achieved either by chemical or biocatalysis. Carboxylic acids having a chiral center and an acidic proton at the α-carbon can be racemized on stage of their esters. Racemization occurs under slightly alkaline conditions, while the free carboxylic acid formed is chirally locked under these conditions. The degree of stabilization of the enol intermediate and, hence, the rate of racemization depend on the nature of substituents at the chiral center and the reactivity of the ester as well. This racemization approach has been utilized by the first study directed to dynamic kinetic resolution to achieve deracemization of **40** using *S. griseus*



(**40**)

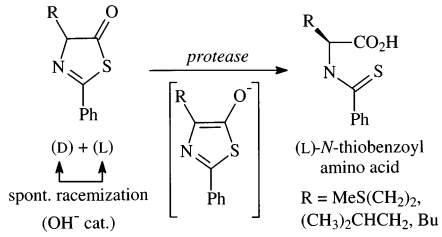
protease as the biocatalyst.[390] At pH = 9.7, the hydrolyzed *S*-enantiomer of **40** could be obtained in 85% *ee* and 92% isolated yield. Current research focuses on the use of thioesters instead of the corresponding oxo esters. Their benefits lie in the higher acidity of the α-hydrogens which leads to higher rates of racemization. Originally, this concept has been used for lipase-catalyzed deracemization of the ethyl thioester of α-(phenylthio)propionate.[391] Later, the same ester of a similar carboxylic acid, i.e., α-phenylpropionate (**41a**), was successfully resolved by



(**41a**) R = CH_2CH_3
(**41b**) R = $CH_2C{\equiv}CH$
(**41c**) R = CH_2CF_3

subtilisin.[392] Significant improvements of the enantioselectivity as well as the degree of conversion could be obtained by using the corresponding propargyl (**41b**) and trifluoroethyl (**41c**) thioesters leading to *ee*'s and conversions of about 80 and 95%, respectively. In situ racemization of α-amino acids can also be achieved by using 4-substituted 2-phenylthiozolin-5-ones as the starting material (Scheme 28). Chrich et al. could show that a number of protease, such as

Scheme 28. Protease-Mediated Dynamic Kinetic Resolution of α-Amino Acids Using 4-Substituted 2-phenylthiazolin-5-ones as the Starting Material



chymotrypsin, protease 2A, or Prozyme 6, are capable of hydrolyzing those substrates leading to the formation of the corresponding L-*N*-thiobenzoyl amino acids in excellent chemical and optical yield.[393] The latter can be chemically converted without racemization into the corresponding *N*-benzoyl analogues. An elegant approach to the in situ racemization of α-amino acid esters has been recently published by Chen et al.[394] The authors used pyridoxal 5-phosphate to achieve racemization of the remaining unhydrolyzed D-enantiomer of the amino acid ester. This approach occurs via a Schiff-base intermediate which facilitates racemization through proton migration. Importantly, pyridoxal 5-phosphate reacts only with the amino acid ester, but not with the amino acid as the product of resolution. The synthetic utility of this approach has been verified in model reactions using alcalase from *Bacillus licheniformis*—a crude protease mixture containing mainly subtilisin—as the biocatalyst. The product L-amino acids, i.e., L-phenylalanine, L-tyrosin, L-leucine, L-norleucine, and L-valine, could be obtained in excellent chemical and optical yield.

Alcohols can also be resolved by dynamic approaches. For example, in situ racemization of secondary alcohols can be achieved via decomposition of labil compounds possessing a secondary alcohol moiety such as a cyanohydrin[395] or hemi(thio) acetal.[396] Alternatively, the unreacted alcohol can be racemized by its temporary oxidation, mediated by a suitable transition metal catalyst.[397] Similarly, the racemization of amines is catalyzed by palladium catalysts.[398] Application of these approaches to synthesis, however, is presently limited to lipases. Therefore, no further attention will be paid to this field, although a general relevance for proteases can be expected, too.

V. Conclusions

Proteases historically and presently represent biocatalysts of outstanding interest in synthetic organic chemistry. It could be demonstrated that after establishing the optimal synthesis conditions, multigram, kilogram, and even ton amounts of natural products or complex synthetics can be obtained by protease catalysis. Although research on improved, it is still the biocatalyst that presents the most interesting target for improvements. Development of appropriate activities and selectivities enable biocatalytic processes in the first place, and improvements of activity and stability may make a process economically feasible. Thus, it is not surprising that

research efforts have focused on improving enzyme properties such as substrate range and specificity, stability, and function. In particular, biocatalysis in organic solvents entails many of the same requirements and issues as standard organic chemical processes with respect to equipment, piping, chemicals handling, storages, safety—all of which are already familiar to the organic chemist. As this review shows, the basic tools to overcome the original low activity and stability of proteases in organic solvents or aqueous−organic mixtures are abundantly available. Current research is now focused on the design of strategies that might restore full enzymatic activity. To take full advantage of such strategies, particular efforts are needed in parallel to develop a generally applicable, quantitative rationale for the solvent dependence of enzymatic selectivities. Besides improving the efficiency of proteases in organic solvents, further investigations should focus on improvements of the environmental compatibility of the solvent system. Already now the toxicity of numerous organic solvents limits the synthetic utility of organic media especially for application in the food sector. There are predominantly processes in aqueous reaction systems, such as the production of aspartame by DSM or LH−RH by Roche, which have found large-scale industrial application. While for the resolution of racemic carboxylic acids, alcohols, and amines or the desymmetrization of prochiral and meso compounds proteases are now generally recognized as normal bench reagents, a general approach to enzymatic polypeptide synthesis still remains to be formulated. However, building on promising strategies, such as site-specific chemically modified enzymes and the substrate mimetics concept, significant improvements can be expected soon. The final breakthrough may be reached by combination of those strategies. Additional input can be expected from the combination of enzyme engineering with directed evolution and gene-shuffling techniques which presently appear to be the most fertile approaches to the design of proteases with tailored selectivities and synthetically relevant activities in essentially any suitable reaction medium. But rational enzyme design, particularly in combination with computational techniques and the de novo design, is also expanding and may contribute to the improvement of proteases for synthesis significantly. The lack of knowledge to predict long range structural changes, which still remains as one of the main hindrances associated with this technology, may be partly compensated by the growing number of enzymes that become systematically studied. Structure−function alignments will be possible whereas the first examples related to proteases, e.g., the isolated thioesterase domain of tyrocidine synthetase which exclusively catalyzes the coupling of peptide bonds without unwanted hydrolytic activity,[399] have been already reported.

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