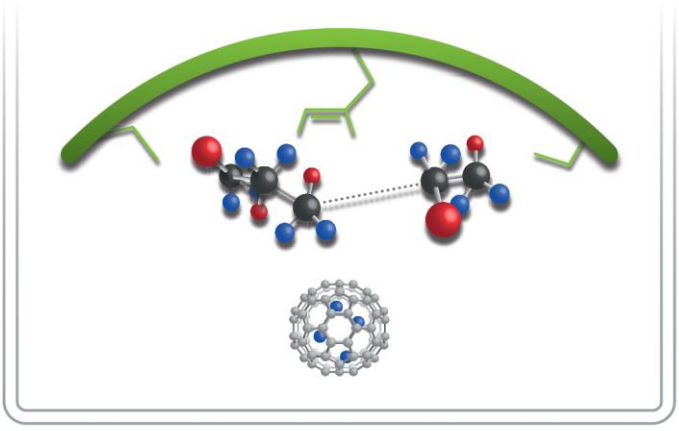
Chemo-Enzymatic Peptide Synthesis

Bioprocess Engineering Aspects



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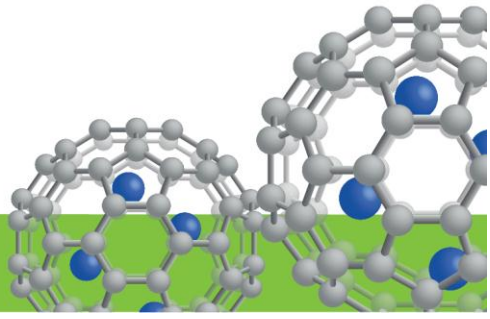

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Chemo-Enzymatic Peptide Synthesis

Bioprocess Engineering Aspects

Petra Vossenberg

Thesis

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Contents

CHAPTER 1

Introduction

1.1 Relevance of peptides

Peptides, in particular oligopeptides, play an important role in the fields of health care, nutrition and cosmetics (Vulfson 1993; Lintner & Peschard 2000; Guzmán *et al.* 2007; Vivó-Sesé & Pla 2007; Stevenson 2009; Quaedflieg *et al.* 2010). Especially pharmaceutical peptides have gained attention in recent years. This interest is caused by the development of novel drug delivery technologies and the applications of peptides in therapeutics against *e.g.* cancer, cardiovascular diseases, infections, and metabolic disorders (Lien & Lowman 2003; News-Medical.Net 2005; Guzmán *et al.* 2007). Advantages of peptides over other small molecules are a high biological activity, a high specificity (minimal unspecific binding), and a low toxicity (minimal accumulation in tissues) (Marx 2005; Pichereau & Allary 2005; Ayoub & Scheidegger 2006).

1.2 Synthesis of peptides

Chemical synthesis is currently the most mature technique for the synthesis of peptides that range in length from 5 to 80 amino acids (Andersson *et al.* 2000; Guzmán *et al.* 2007; Thayer 2011). Such a length is a typical size for therapeutic peptides (PharmPro 2009). Chemical synthesis is, however, expected to be more and more combined with enzyme-catalyzed synthesis, resulting in chemo-enzymatic approaches towards peptide synthesis (Hou *et al.* 2005; Guzmán *et al.* 2007). The racemization that hampers chemical synthesis can be prevented by forming the peptide bond enzymatically (Fruton 1982; Gill *et al.* 1996; Kumar & Bhalla 2005).

In the Dutch national research program "Integration of Biosynthesis and Organic Synthesis" (IBOS) a chemo-enzymatic route for the synthesis of peptides is proposed. In this stepwise route two amino acids, or a peptide and an amino acid, or two peptides, are coupled with a protease. An N-protected, C-terminally activated amino acid / peptide is coupled with a C-protected amino acid / peptide nucleophile. The peptide synthesis is done in the N → C-terminal direction (*i.e.* C-terminal extension). After the coupling, the C-terminus of the product is

deprotected and then activated with a lipase or peptide amidase (Figure 1.1) (Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011).

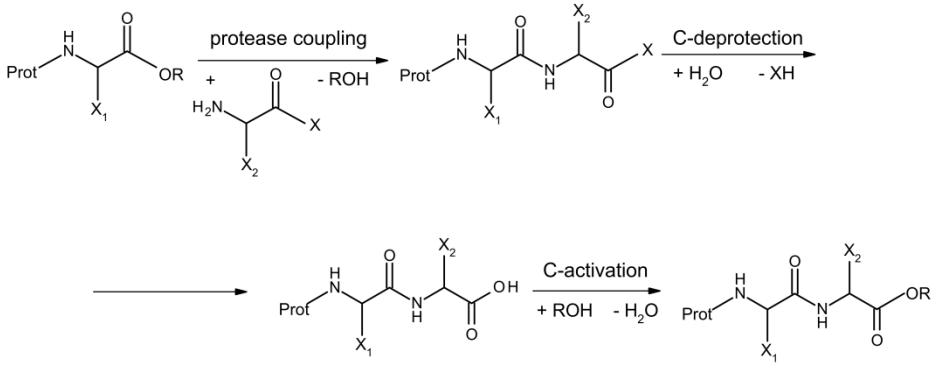

Figure 1.1. Coupling of an N-protected, C-terminally activated amino acid with a C-protected amino acid nucleophile with a protease and subsequent C-deprotection and C-activation. X_1 and X_2 can be any amino acid side chain. R is an activating group (for example an alkyl ester moiety). X is a C-protection group (for example NH_2).

The suggested protease-catalyzed coupling is a kinetically controlled process, in which the product concentration passes through a maximum before the slower hydrolysis of the product becomes significant (Sewald & Jakubke 2002b). Kinetically controlled syntheses can only be carried out by using proteases that can form acyl-enzyme intermediates, *i.e.* serine and cysteine proteases (Fruton 1982; Bordusa 2002), because the enzyme acts as a transferase catalyzing the transfer of an acyl group from the acyl donor to the amino acid nucleophile (Guzmán *et al.* 2007). The kinetic approach requires the use of an acyl donor ester, *i.e.* an activated ester, as the carboxyl component (Nuijens *et al.* 2012). The presence of water can lead to undesired hydrolytic side reactions, such as the hydrolysis of the activated substrate or of the product (Kasche 1986). A high ratio between wanted and unwanted reactions, *i.e.* a high synthesis / hydrolysis (S/H) ratio, is of importance for successful peptide synthesis.

Different aspects of the suggested chemo-enzymatic route for the synthesis of peptides were studied by different groups in the IBOS-2 research project 'Chemo-Enzymatic Peptide Synthesis'. At the University of Groningen, research focused on new and improved enzymes for the activation, coupling, and deprotection steps. The ultimate goals were to find an enzyme that could both deprotect and activate,

and to find a protease with a high S/H ratio, which could be used in a partially aqueous solution. At the Radboud University Nijmegen, research aimed at overcoming inadequate substrate recognition by synthesis of new activating groups for acyl-enzyme formation and at the testing of these compounds with different commercially available enzymes such as chymotrypsin (serine protease), subtilisin (serine protease), trypsin (serine protease), and papain (cysteine protease) (Sigma-Aldrich 2010; Worthington Biochemical Corporation 2012). The ultimate goals here were to overcome the generally narrow substrate specificities of enzymes, in particular the exclusion of unnatural amino acids, and minimize undesired hydrolysis reactions under aqueous conditions, using substrate engineering (De Beer *et al.* 2011; De Beer *et al.* 2012a, De Beer *et al.* 2012b, De Beer *et al.* 2012c).

1.3 Research aim

The aim of this thesis is to investigate the bioprocess engineering aspects of the proposed chemo-enzymatic route, in which Alcalase was used as the protease to catalyze the coupling step. Alcalase may also be referred to as subtilisin A or subtilisin Carlsberg (De Lange & Smith 1968; Ottesen & Svendsen 1970; Gupta *et al.* 2002; Sigma-Aldrich 2010). The focus is on evaluating and optimizing the compatibility of Alcalase with the process conditions (Chapters 2 - 7), leading to a process design (Chapter 8).

1.4 Thesis outline

Chapter 2 describes the incompatibility between lipase B from *Candida antarctica* (CalB) and Alcalase. These enzymes were a model system for one-pot chemo-enzymatic peptide synthesis, in which the lipase would catalyze the deprotection and the activation of the C-terminus and the protease would catalyze the coupling reaction (Figure 1.1). It was shown that native Alcalase degrades native CalB under aqueous conditions. Immobilization of both or either CalB or Alcalase onto macroporous beads, however, effectively prevented hydrolysis of CalB by Alcalase. As research at DSM (Quaedflieg & Nuijens 2009, personal communication) showed that the activation step is currently more feasible using chemical instead of

enzymatic methods, the Wageningen research focused on the protease-catalyzed coupling step. The non-aqueous coupling in tetrahydrofuran (THF) of phenylalanine amide (Phe-NH_2) and the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam) was used as a model reaction.

Chapter 3 describes the coupling reaction catalyzed by cross-linked enzyme aggregates of Alcalase optimized for use in organic media (Alcalase CLEA-OM). The coupling was studied at a range of water activities (a_w), in the presence of molecular sieves if needed (*i.e.* at very low a_w values), to investigate up to which a_w values the rate of peptide synthesis could be increased without significantly increasing the rate of the hydrolysis. Hydrolysis (in the present system only the activated substrate, not the dipeptide product, may be hydrolyzed) was found to dominate above $a_w \approx 0.2$. To prevent hydrolysis, the presence of molecular sieves was found to be necessary. Nevertheless, the use of molecular sieves over longer periods of time should be carefully considered as they may dehydrate and thereby inactivate the enzyme in time.

Chapter 4 describes the use of different Alcalase formulations for catalyzing the coupling reaction in the presence of molecular sieves (*i.e.* under near-dry conditions). Hydration prior to drying (with anhydrous *tert*-butanol and anhydrous THF) of the Alcalase formulations resulted in a significant increase in rate of the subsequent dipeptide synthesis. Repeated use in the presence of molecular sieves, without intermediate rehydration, led to inactivation of the enzyme. For three enzyme formulations this inactivation could be counteracted by intermediate rehydration. Inactivation of another enzyme formulation, Alcalase immobilized onto dicalite, was only partially reversible by hydration. Alcalase immobilized onto dicalite was found to be initially the most active in dipeptide synthesis. Nevertheless, due to its small particle size and its lack of operational stability, this formulation may not be the best choice for the synthesis of dipeptides in neat organic media on a large scale. The most promising enzyme formulation for this is Alcalase covalently immobilized onto macroporous acrylic beads (in this thesis abbreviated as Cov) due to its reasonable activity, its seemingly good operational stability and its practical size and uniform spherical shape.

In Chapter 5 the long-term stability and reuse of 'hydrated' (water saturated) Cov in THF is described. Without molecular sieve beads Cov hardly inactivated in THF. With molecular sieve beads, however, Cov lost activity over time. Incubated Cov samples were rotated on a blood rotator, entailing mechanical forces between Cov and the molecular sieve beads. Mechanical damage of Cov by the molecular sieve beads was found to be the main reason for the instability of Cov. During reuse, intermediate rehydration of Cov also caused a small but significant activity loss.

Chapter 6 describes the kinetics of the coupling reaction catalyzed by hydrated Cov in the presence of a carefully chosen amount of molecular sieve powder. Kinetic characteristics were determined from reaction time courses up to full conversion at various initial concentrations of substrate and product. It was found that the kinetics of the coupling can be described well with a two-substrate kinetic model with two inhibitory products.

In Chapter 7 the observed (Chapter 4) effect of enzyme dehydration by molecular sieves on the Cov-catalyzed coupling reaction is described in detail. Dehydration-induced inactivation of Cov by molecular sieve powder was found to occur in three phases: (1) an initial, rapid, major dehydration-induced inactivation that takes place during the first activity measurement (1 h), (2) a phase of first-order inactivation (20 h), and (3) a relatively low plateau phase in activity. These dehydration kinetics were incorporated into the reaction kinetics model described in Chapter 6. The resulting model was then used to fit progress curve data of the coupling in the presence of different amounts of molecular sieve powder. Using the estimated parameter values, the model was used to predict independent data sets and found to work well.

Chapter 8 concludes the thesis with a general discussion. In the general discussion a process design for chemo-enzymatic peptide synthesis is proposed based on the findings of the previous chapters. The choices with regard to Alcalase formulation, type of reactor, way to control the water content, and whether or not to recycle the enzyme, are discussed. In addition, an estimate is given for the reactor size, volumes of solvent, amounts of substrate, enzyme and molecular sieves, needed in order to produce a specific demand for peptides.

CHAPTER 2

Immobilization to prevent enzyme incompatibility with proteases

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. 2011. Immobilization to prevent enzyme incompatibility with proteases. Biocatalysis and Biotransformation 29:288-298.

Abstract

Enzyme incompatibility is a problem in multi-enzyme processes that involve a non-specific protease, such as Alcalase. An example is the one-pot enzymatic synthesis of peptides catalyzed by a lipase and a protease. The incompatibility between lipase B from *Candida antarctica* (CalB) and Alcalase was studied. To what extent immobilization of both or either CalB or Alcalase onto macroporous beads helps to prevent hydrolysis of CalB by Alcalase was evaluated. The rate of activity loss of native and immobilized CalB in the absence and presence of native and immobilized Alcalase was calculated from the rate of triacetin hydrolysis. Immobilization of both or either CalB or Alcalase onto macroporous beads was found to be effective in largely preventing hydrolysis of CalB by Alcalase.

Keywords: *Candida antarctica lipase B, Alcalase, subtilisin Carlsberg, proteolysis, CalB*

2.1 Introduction

Natural and non-natural peptides are important for pharmaceutical and food applications (Guzmán *et al.* 2007). These peptides may be synthesized enzymatically. In the Dutch national research program "Integration of Biosynthesis and Organic Synthesis" (IBOS) an enzymatic route for the synthesis of peptides is proposed. The C-terminus of an N-protected amino acid is activated with a lipase. Subsequently, a protease couples this activated amino acid with an amino acid nucleophile to give a dipeptide (Figure 2.1) (Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011). The activation step is an equilibrium process. To shift the equilibrium towards synthesis of peptides, activation and coupling of the amino acids should be done in one pot.

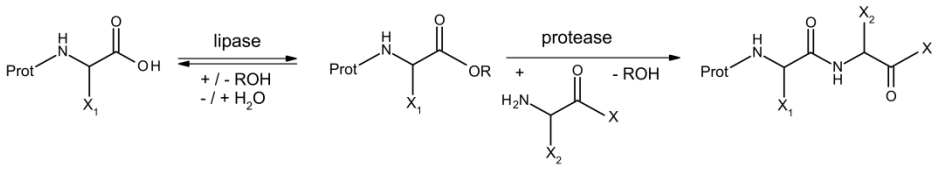

Figure 2.1. Activation of N-protected amino acid using a lipase and subsequent coupling with an amino acid nucleophile using a protease to give a dipeptide. X_1 and X_2 can be any amino acid side chain. R is an activating group (for example an alkyl ester moiety). X is a C-protection group (for example NH_2).

Currently, the one-pot system is carried out under dry conditions (*i.e.* in organic solvent containing molecular sieves). However, there is still an optimization in water activity possible as enzymes need some water to maintain their catalytically active conformation (Gaertner & Puigserver 1989; Adlercreutz 1991; Bordusa 2002). With an increase in water activity, however, an enzyme incompatibility problem may be introduced. The protease will not only couple desired amino acids but also hydrolyze and thereby inactivate the lipase.

This problem of enzyme incompatibility is also relevant in other multi-enzyme processes and products that involve a non-specific protease, such as the combination of different enzymes in liquid detergents (Stoner *et al.* 2004). Several options exist to minimize or prevent proteolysis: adding new enzyme to

compensate proteolytic losses, genetically modifying the protease (Bae *et al.* 1995), and adapting process conditions, such as lowering the water activity by adding organic solvents (Wangikar *et al.* 1997). An obvious but, to our knowledge, not yet studied option is enzyme immobilization.

As a model system, the incompatibility between lipase B from *Candida antarctica* (CalB) and Alcalase (protease from *Bacillus licheniformis)* was studied. CalB and Alcalase were chosen as model enzymes because CalB is one of the most used lipases, due to its high activity and stability (Torres *et al.* 2006), and Alcalase is a non-specific protease (Tardioli *et al.* 2003) used abundantly in the detergent industry (Maurer 2004). This non-specificity should lead to a significant degradation of any protein, including Alcalase itself (Colleary & Ó'Fágáin 2008) and CalB.

Five enzyme formulations were used: (1) native CalB, (2) CalB covalently immobilized or (3) adsorbed onto macroporous acrylic beads, (4) native Alcalase, and (5) Alcalase covalently immobilized onto macroporous acrylic beads. Immobilization onto macroporous acrylic beads was chosen as the immobilization method because CalB and Alcalase are commercially available in this form, the beads are highly porous so mass transfer limitations should be minimal for small molecules, and the beads are mechanically stable.

The aim of our study was to evaluate whether immobilization of both or either CalB or Alcalase onto macroporous beads would help to prevent hydrolysis of CalB by Alcalase. The activity of CalB was assayed by continuously following the hydrolysis of triacetin. The change in the rate of triacetin hydrolysis over time served as a measure of the loss of activity of native or immobilized CalB in the absence and in the presence of native or immobilized Alcalase.

2.2 Materials and methods

2.2.1 Enzymes

Native and immobilized CalB, produced recombinantly in *Aspergillus oryzae*, and protease from *Bacillus licheniformis* (also referred to as Alcalase®, Subtilisin® A, and Subtilisin® Carlsberg) were purchased from Sigma-Aldrich (Zwijndrecht, The

Netherlands). Immobilized Alcalase and a second immobilized CalB formulation were obtained from ChiralVision (Leiden, The Netherlands).

The approximate dimensions of CalB are 3 x 4 x 5 nm and it has a molecular weight of about 33 kDa (Uppenberg *et al.* 1994). The approximate dimensions of the protease from *Bacillus licheniformis* are 5 x 6 x 8 nm (Schmitke *et al.* 1997) and it has a molecular weight of about 27 kDa (Sigma-Aldrich 2010).

Immobilized CalB (Novozym® 435) purchased from Sigma-Aldrich is a product from Novozyme Corporation (Bagsvaerd, Denmark). The lipase is adsorbed on Lewatit E, which is a macroporous acrylic ion-exchange resin (anionic) (Soledad de Castro & Sinisterra Gago 1998) from Bayer Corporation (Pittsburgh, USA). The bead-shaped particles have a diameter in the range of 300 to 900 μm, and the pore diameter of these beads averages approximately 15 nm (information obtained from supplier).

The formulations from ChiralVision contain enzymes from Novozyme Corporation, covalently immobilized onto Immobeads (crosslinked copolymer of methacrylate carrying oxirane groups) produced by ChiralVision in cooperation with FermentaBiotech Ltd. (Maharashtra, India). The Immobeads have a diameter in the range of 150 to 300 μm, and the pore diameter of these beads averages approximately 70 nm (information obtained from supplier).

2.2.2 Chemicals

All chemicals used were reagent or analytical grade.

2.2.3 CalB activity

The activity of CalB was assayed by following the hydrolysis of triacetin at pH 7.5. The acetic acid that is formed was titrated with 0.1 mol l^{-1} sodium hydroxide using pH-stat equipment (719 Stat Titrino Metrohm; Herisau, Switzerland). The pH-stat equipment was connected to a computer that logged the consumption of sodium hydroxide every two seconds.

The activity of CalB was studied at a pH of 7.5, which is a compromise between the optimal pH's for catalysis of CalB and Alcalase. The optimal pH for catalysis of CalB is 7, but the enzyme is stable in aqueous media in the range of pH 3.5 to 9.5

(Anderson *et al.* 1998). The optimal pH for catalysis of Alcalase is between 8 and 9 (Uhlig 1998).

In a thermostatted vessel at 40 °C, 25 ml Milli-Q, 10 ml sodium phosphate buffer (0.1 mol l^{-1}), and 2 ml triacetin were incubated. The initial triacetin concentration was 0.28 mol l^{-1}. After pH and temperature stabilization (about 30 minutes), CalB was added. One unit of triacetin activity (TAU) is defined as the amount of enzyme that produces 1 µmol of acetic acid per minute at pH 7.5 and 40 °C.

Other common methods to determine the activity of CalB are based on the hydrolysis of tributyrin, para-nitrophenyl butyrate, or para-nitrophenyl palmitate. Triacetin was preferred over tributyrin because the former is better soluble in water and therefore the formation of an emulsion can be avoided.

2.2.4 Kinetic analysis

To estimate the (apparent) Michaelis constant K_m (K'_m), and the (apparent) maximum specific reaction rate v_{max} (v'_{max}) of (immobilized) CalB, the triacetin concentration was varied from 0.017 mol l^{-1} to 0.27 mol l^{-1}. The maximum concentration of triacetin in water at 25 °C is 0.24 mol l^{-1} (Lide 2001). At higher concentrations an emulsion is formed. The CalB activity assay was carried out at 40 °C and therefore a slightly higher triacetin concentration could be used without formation of an emulsion. The initial reaction rate of native CalB and the two immobilized CalB formulations was determined as described above. In addition, the Michaelis-Menten parameters of native Alcalase with the substrate triacetin were estimated because Alcalase has a relatively small but significant triacetin hydrolysis activity. The parameters were estimated by using nonlinear regression.

2.2.5 Alcalase activity

The activity of Alcalase was assayed by following the hydrolysis of 25 % (v/v) ethyl lactate at 40 °C and pH 6.8 (10 ml of 100 mM sodium phosphate buffer pH 6.8, 20 ml of Milli-Q, and 10 ml of ethyl lactate). The lactic acid that is formed was titrated with 0.1 mol l^{-1} sodium hydroxide and the consumption of the latter was recorded as a function of time. One unit of ethyl lactate activity (ELU) is defined as the amount of enzyme that produces 1 µmol of lactic acid per minute at

pH 6.8 and 40 °C. The protocol was obtained from ChiralVision (ChiralVision 2009).

2.2.6 Online measurement of CalB activity

Nine enzyme combinations were made (Table 2.1). These combinations were added to a thermostatted vessel at 40 °C in which a triacetin assay took place, as described above. The CalB activity was followed over time. The slope of the graph of sodium hydroxide addition against time was determined from 100 data points in a 200 second interval. The slope is one data point representing the CalB activity at a specific time.

The amount of immobilized CalB added to the triacetin assay was based on the activity measurement of native and immobilized CalB with triacetin. The average initial CalB activity in the assays was 17000 TAU with a deviation of ± 18 %. The amount of immobilized Alcalase added to the triacetin assay was based on the activity measurement of native and immobilized Alcalase with ethyl lactate. About 54 mg of immobilized Alcalase (enzyme and carrier) had the same activity as 1 mg of native Alcalase.

Table 2.1. Overview of enzyme combinations of which the activity of CalB was followed over time.

	No Alcalase	Native Alcalase	Immobilized Alcalase
Native CalB	✓	✓	✓
Adsorbed CalB	✓	✓	✓
Covalently immobilized CalB	✓	✓	✓

2.2.7 SDS-PAGE

Native CalB (30 mg/ml) was incubated with different amounts of native Alcalase (0.9 - 3.7 mg/ml) in Milli-Q for 24 hours at 25 °C. Native CalB (30 mg/ml) and the different amounts of native Alcalase (0.9 - 3.7 mg/ml) were also incubated separately. After 24 hours the different samples were diluted 1:1 with sample buffer (Sigma-Aldrich) containing 100 mM Tris-HCl (pH 6.8), 1 % SDS, 4 % β-mercaptoethanol, 0.02 % Brilliant Blue G, and 24 % glycerol. An Ultra-Low Range Molecular Weight Marker (Sigma-Aldrich) was diluted with sample buffer that was

diluted 2-fold with Milli-Q. The samples were heated for 5 minutes at 95 °C. After cooling, the samples were loaded (10 µl/well) to a 16.5 % Tris-Tricine gel (Bio-Rad Laboratories). The gel was run at 100 V for 105 minutes at 25 °C with Tris-HCl (100 mM, pH 8.3, 0.1 % SDS) (Bio-Rad Laboratories) as running buffer. The gel was placed for 30 minutes in polypeptide fixative solution containing 40 % (v/v) methanol, 10 % (v/v) acetic acid, and 50 % (v/v) Milli-Q. The gel was stained in Coomassie Blue G-250 Staining Solution (Bio-Rad Laboratories) for 1 hour and destained for 3 x 15 minutes in Coomassie Blue G-250 destaining solution containing 10 % (v/v) acetic acid and 90 % (v/v) Milli-Q.

2.3 Results and Discussion

The rate of triacetin hydrolysis was taken as a measure of the activity of native and immobilized CalB. Any change in this rate over time was assumed to reflect activity loss of native or immobilized CalB, both in the absence and in the presence of native or immobilized Alcalase. The triacetin concentration, however, decreases during the assay due to reaction and dilution. To assess the effect of this change on the triacetin hydrolysis rate, the (apparent) Michaelis constant K_m (K'_m) and the (apparent) maximum specific reaction rate v_{max} (v'_{max}), were estimated for the different CalB formulations (Table 2.2). In addition, the triacetin hydrolysis rate not only depends on the concentration of active CalB, but also on the concentration of Alcalase: like CalB, Alcalase hydrolyzes triacetin. The triacetin activities of native Alcalase, of immobilized Alcalase, and of native CalB were measured separately. These measurements show that the hydrolytic activity of native CalB is about 6x higher per gram than that of native Alcalase and about 90x higher per gram than that of immobilized Alcalase (own data). When native CalB and native Alcalase were combined, the initial triacetin hydrolyzing activity of Alcalase was at most 5 % of that of CalB and can therefore be disregarded. When native CalB and immobilized Alcalase were combined, the initial activity of Alcalase was about 15 % of that of CalB. When immobilized CalB and native Alcalase were combined, the initial activity of Alcalase was at most 30 % of that of CalB. This significant additional hydrolysis of triacetin by Alcalase would not matter if it would stay constant. However, the rate of triacetin hydrolysis by Alcalase does change

because of a changing triacetin concentration. In addition, the rate of triacetin hydrolysis by Alcalase can change due to Alcalase inactivation. To assess the effect of a changing substrate concentration on the triacetin hydrolysis rate by Alcalase, the K_m and v_{max} of native Alcalase (Table 2.2) were estimated. Alcalase itself does not show any inactivation in the triacetin hydrolysis assay for about 4 hours under the experimental conditions.

Table 2.2. (Apparent) Michaelis constant K_m (K'_m) and (apparent) maximum specific reaction rate v_{max} (v'_{max}) of different CalB formulations and native Alcalase with the substrate triacetin.

	K_m (K'_m) (M)	v_{max} (v'_{max}) (TAU/g enzyme formulation)
Native CalB	0.044 ± 0.038	5600 ± 1400
Adsorbed CalB	0.014 ± 0.011	350 ± 50
Covalently immobilized CalB	0.042 ± 0.017	450 ± 50
Native Alcalase	0.28 ± 0.23	1400 ± 700

To assess the activity loss of CalB itself, native or immobilized, in the different enzyme combinations, the overall change in triacetin hydrolysis rate was corrected for the change in rate due to a decrease in measured substrate concentration. All experimental data were corrected using the Michaelis-Menten data of CalB. Only the experimental data of the two worst cases, in which the initial activity of Alcalase was about 30 % of the initial activity of CalB, were corrected for the contribution by Alcalase by using the Michaelis-Menten data of Alcalase. For lower percentages of Alcalase triacetin activity compared to CalB activity, this correction was considered superfluous.

Figure 2.2 serves as an example of how two sets of experimental data were affected due to correction using the Michaelis-Menten simulation of CalB. It can be seen that in the case of native CalB, the change in the triacetin hydrolysis rate due to a decrease in substrate concentration (Michaelis-Menten simulation) is similar to the measured change in the triacetin hydrolysis rate (experimental data). In this case the correction of the experimental data is significant. In the case of native CalB and 0.5 mg Alcalase together, it can be seen that difference between the raw

experimental data and the corrected data is almost negligible. The change in the triacetin hydrolysis rate due to a decrease in substrate concentration is minimal compared to the measured change in the triacetin hydrolysis rate in the case of native CalB and 0.5 mg Alcalase.

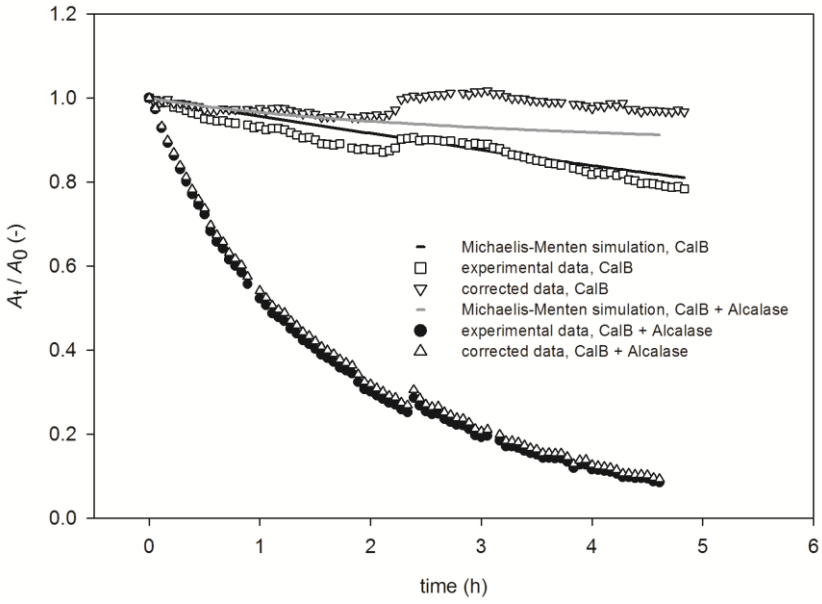

Figure 2.2. Change in the rate of triacetin hydrolysis estimated using Michaelis-Menten data of CalB and measured substrate concentrations (Michaelis-Menten simulation), change in the rate of triacetin hydrolysis measured in the experimental set-up (experimental data), and experimental data corrected using the Michaelis-Menten simulation for native CalB, and native CalB and 0.5 mg Alcalase.

After correction for the decrease in activity due to the decrease in substrate concentration, the remaining loss of activity of CalB in the different enzyme combinations can be compared. A quantitative comparison was facilitated by describing the loss of enzyme activity by a simple, yet appropriate model. It was chosen to describe the loss of activity of CalB using a simple exponential decay model containing a minimum number of parameters.

Some of the experimental data could be fitted well with a single exponential decay function,

$$\frac{A_t}{A_0} = e^{-kt} \tag{2.1}$$

where A_t is the enzyme activity (activity units) at time t (h), A_0 is the initial enzyme activity (activity units), and k is the inactivation rate constant (h^{-1}).

Most of the experimental data with immobilized CalB could, however, be fit better with a double exponential decay function (Henley & Sadana 1986; Ulbrich *et al.* 1986; Aymard & Belarbi 2000),

$$\frac{A_t}{A_0} = x_1 e^{-k_1 t} + x_2 e^{-k_2 t} \tag{2.2}$$

where x_1 is the fraction of enzyme population A_1, k_1 is the inactivation rate constant of A_1 (h^{-1}), x_2 (x_2 = 1- x_1) is the fraction of enzyme population A_2, and k_2 is the inactivation rate constant of A_2 (h^{-1}). The double exponential decay function is actually a simplification from a more general enzyme decay function that assumes a continuous distribution of inactivation rates (Henley & Sadana 1986; Ulbrich *et al.* 1986). The occurrence of several enzyme populations is not unlikely for immobilized enzymes because there is a heterogeneity in enzyme orientation, location of the enzyme in the carrier, and number of linkages between the enzyme and the carrier (Ulbrich *et al.* 1986). An infinite number of parameters is, however, not practical to compare. The general decay function should thus be simplified to a form that gives an acceptable fit of the data but keeps the number of fit parameters to a minimum. It was therefore simplified to a double exponential decay function.

In literature the thermal inactivation of native and adsorbed CalB on Lewatit E, at 50 °C in 100 mM Tris-HCl buffer pH 8, has been reported to follow single exponential decay, whereas the thermal inactivation of adsorbed CalB on Lewatit OC 1600 and covalently immobilized CalB has been reported to follow double exponential decay (Arroyo *et al.* 1998; Arroyo *et al.* 1999).

The single and double exponential decay functions were used to fit the degradation of native (Figure 2.3), covalently immobilized (Figure 2.4), and adsorbed CalB (Figure 2.5), in the absence and presence of native Alcalase or immobilized Alcalase. The resulting parameters, k, x_1, k_1, x_2, k_2, and the R^2 of the fits are listed in Table 2.3. The values for k, x_1, k_1, x_2, and k_2, that yielded an R^2 of 0 - 0.1, were omitted from Table 2.3.

In Figures 2.3, 2.4, and 2.5, CalB appears to more or less suddenly regain some activity at a certain stage in time. The reason for this is unclear.

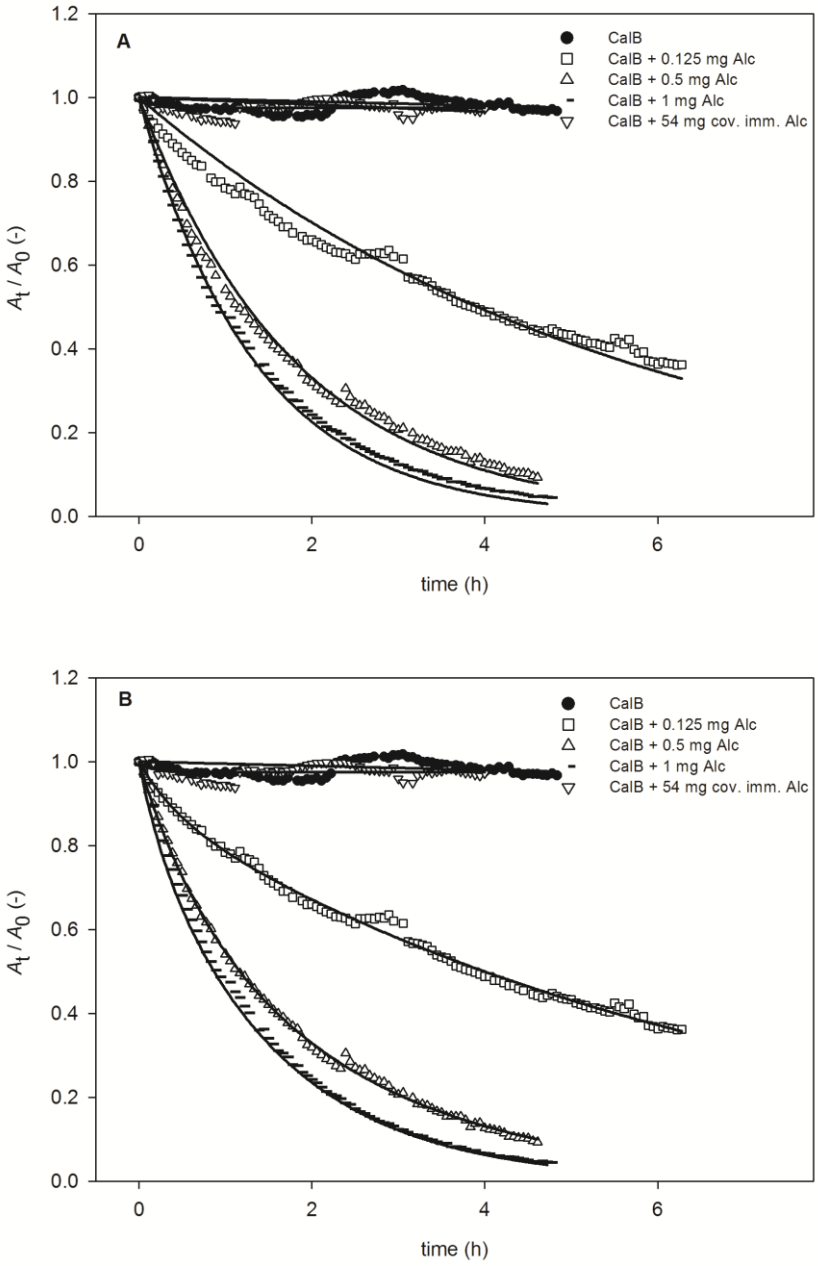

Figure 2.3. Degradation of native CalB in the absence and in the presence of native or covalently immobilized Alcalase. The data points are fitted using a single exponential decay function (**A**) and using a double exponential decay function (**B**).

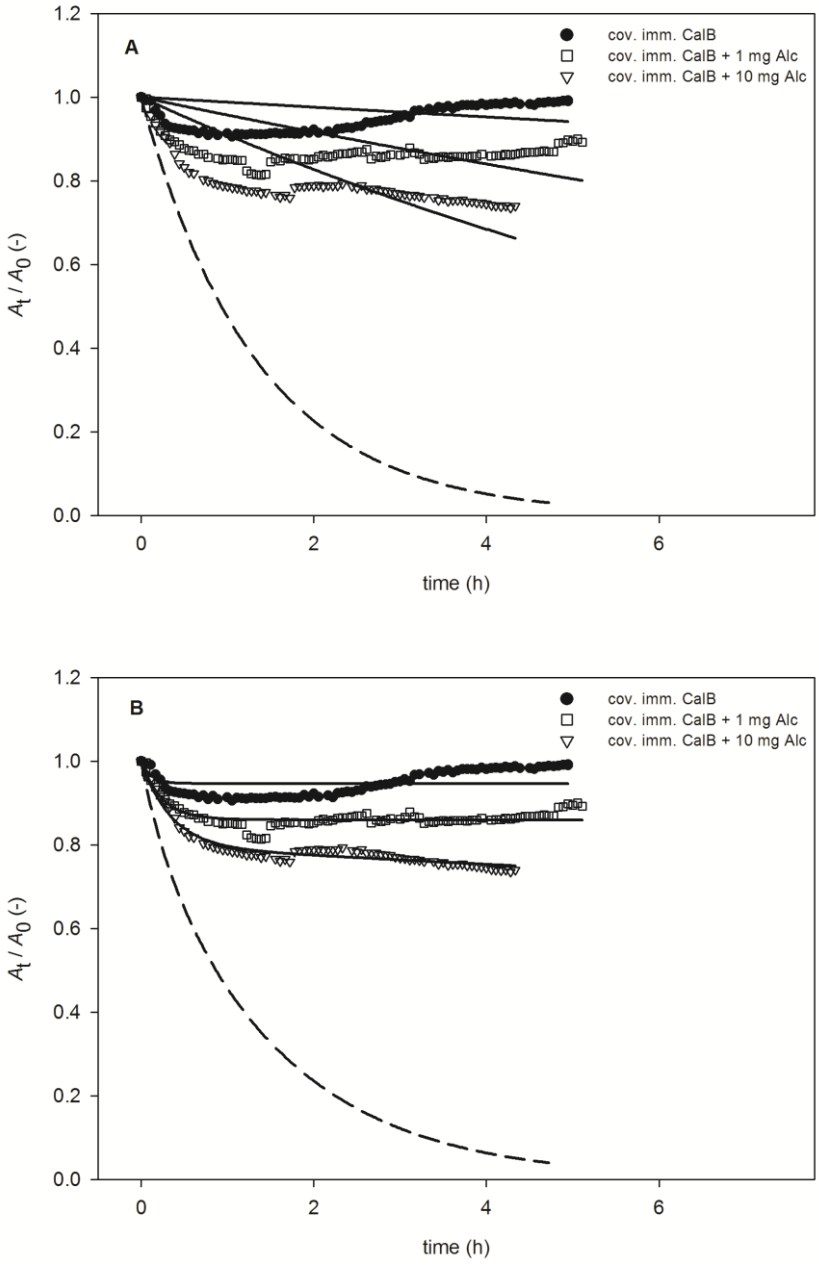

Figure 2.4. Degradation of covalently immobilized CalB in the absence and in the presence of native Alcalase. The data points are fitted using a single exponential decay function (**A**) and using a double exponential decay function (**B**). The degradation of native CalB in the presence of 1 mg of native Alcalase serves as a reference (shown as dashed line).

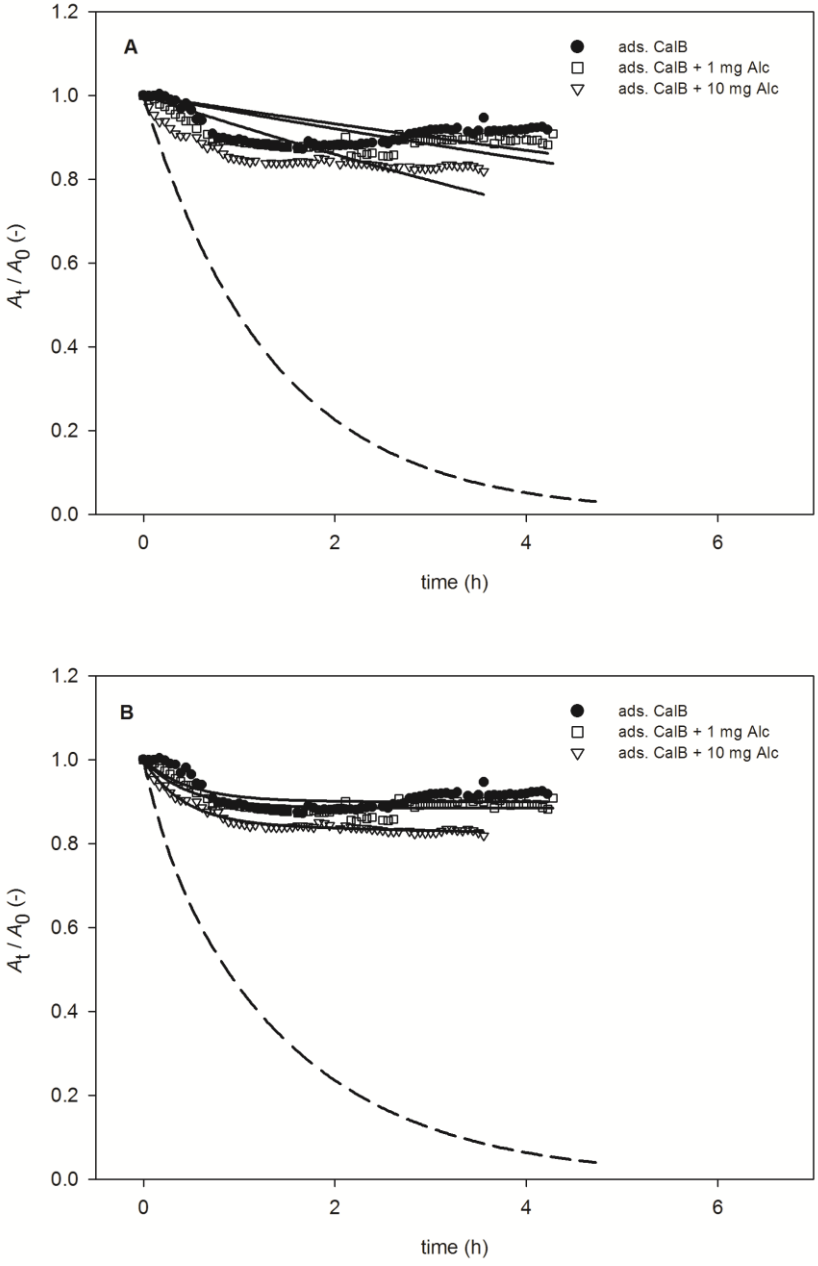

Figure 2.5. Degradation of adsorbed CalB in the absence and in the presence of native Alcalase. The data points are fitted using a single exponential decay function (**A**) and using a double exponential decay function (**B**). The degradation of native CalB in the presence of 1 mg of native Alcalase serves as a reference (shown as dashed line).

Table 2.3. Parameters of the single and double exponential decay functions used to fit the degradation of native, covalently (cov) immobilized and adsorbed CalB (ads), in the absence and presence of native and immobilized Alcalase (Alc).

				Single exponential decay			Double exponential decay					
native CalB (mg)	imm. CalB (mg)	native Alc (mg)	imm. Alc (mg)	k (h^{-1})	R^2	Figure	x_1	k_1 (h^{-1})	x_2	k_2 (h^{-1})	R^2	Figure
4	0	0	0	-	0	2.3 A	-	-	-	-	0	2.3 B
4	0	0.125	0	1.8E-01	0.968	2.3 A	1.0E-01	2.3E+00	9.0E-01	1.5E-01	0.994	2.3 B
4	0	0.5	0	5.5E-01	0.992	2.3 A	2.2E-01	1.5E+00	7.8E-01	4.5E-01	0.999	2.3 B
4	0	1	0	7.4E-01	0.994	2.3 A	1.2E-01	3.9E+00	8.8E-01	6.6E-01	1	2.3 B
4	0	0	54	-	0	2.3 A	-	-	-	-	0.107	2.3 B
0	35 (cov)	0	0	-	0	2.4 A	-	-	-	-	0.072	2.4 B
0	42 (cov)	1	0	-	0	2.4 A	1.4E-01	4.1E+00	8.6E-01	1E-10	0.716	2.4 B
0	42 (cov)	10	0	-	0	2.4 A	2.3E-01	3.8E+00	7.8E-01	2E-10	0.824	2.4 B
0	50 (ads)	0	0	-	0	2.5 A	1.0E-01	2.1E+00	9.0E-01	3E-13	0.636	2.5 B
0	45 (ads)	1	0	-	0	2.5 A	1.2E-01	2.2E+00	8.8E-01	2E-13	0.790	2.5 B
0	45 (ads)	10	0	-	0	2.5 A	1.1E-01	6.1E+00	8.9E-01	2E-10	0.493	2.5 B

Although the degradation of native CalB in the presence of native Alcalase can be fit well with the single exponential decay function (R^2 > 0.968), the double exponential decay function yields a somewhat better fit (R^2 > 0.994) (Figure 2.3 and Table 2.3). Nevertheless, one might argue that the difference between the single and double exponential decay functions fittings are not so significant. It was therefore decided to base the conclusions for native CalB in the presence of native Alcalase on the single exponential decay function fit.

The degradation of immobilized CalB cannot be fit well with the single exponential decay function as indicated by R^2 = 0 (Figure 2.4 A, Figure 2.5 A, and Table 2.3) in all cases. The double exponential decay function can however be used to fit the degradation of immobilized CalB, except for the slight degradation of covalently immobilized CalB in the absence of Alcalase, R^2 ranges from 0.49 to 0.82 (Figure 2.4 B, Figure 2.5 B and Table 2.3). Covalently immobilized CalB in the absence of Alcalase shows no correlation with the double exponential decay function as there is hardly any degradation.

When native CalB is incubated with native Alcalase, a large decrease in triacetin hydrolyzing activity occurs (Figure 2.3 and Table 2.3). The activity decreases with an increasing amount of native Alcalase: k increases from 1.8E-01 h^{-1} with 0.125 mg of Alcalase to 7.4E-01 h^{-1} with 1 mg of Alcalase. As the initial triacetin hydrolyzing activity of native Alcalase was at most 5 % of that of CalB, the large decrease in activity upon incubation of CalB with native Alcalase can only be caused by a decrease in activity of CalB.

To determine whether the slower triacetin hydrolysis seen when incubating native CalB and native Alcalase together is indeed caused by the degradation of CalB, on a protein level, by Alcalase, a SDS-PAGE analysis of native CalB (30 mg/ml) that was incubated with different amounts of native Alcalase (0 - 3.7 mg/ml) in Milli-Q for 24 hours at 25 °C was carried out (Figure 2.6).

When lanes 1 and 2 (solely native CalB) are compared to lanes 3 - 10, it is clear that CalB is indeed degraded to fragments by Alcalase. An increasing amount of Alcalase leads to an increase in peptide fragments. The contribution of Alcalase, as a protein, to these fragments is minimal as can be seen when comparing lanes 3 - 10 with lanes 12 - 15 (solely native Alcalase). Alcalase can hardly be seen in

lanes 12 - 15 due to the low concentration used. This low concentration is, however, high enough to degrade CalB.

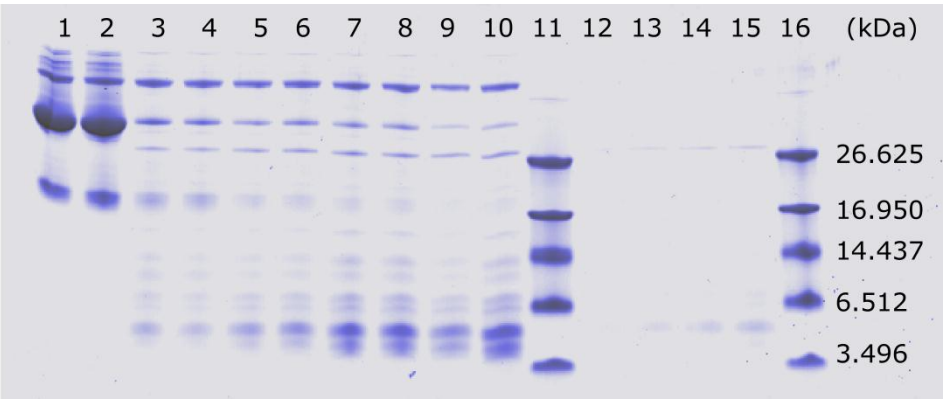

Figure 2.6. SDS-PAGE analysis of native CalB (30 mg/ml) that was incubated with different amounts of native Alcalase (0.9 (lanes 3 and 4), 1.9 (lanes 5 and 6), 2.8 (lanes 7 and 8), and 3.7 mg/ml (lanes 9 and 10)) in Milli-Q for 24 hours at 25 °C. Native CalB (lanes 1 and 2) and the different amounts of native Alcalase (0.9 (lane 12), 1.9 (lane 13), 2.8 (lane 14), and 3.7 mg/ml (lane 15)) were also incubated separately. Lanes 11 and 16 contain a marker.

When comparing the effect of native and immobilized Alcalase on the activity of native CalB (Figure 2.3 and Table 2.3) it can be seen that the degradation of CalB with native Alcalase (57 ELU, 1 mg) is much faster (k = 7.4E-01 h^{-1}) than with immobilized Alcalase (57 ELU, 54 mg). Native CalB in the absence and in the presence of immobilized Alcalase shows no significant decrease in activity for about 4 hours and therefore also shows no or hardly any correlation with the single nor the double exponential decay function. Native CalB in the presence of native Alcalase loses its activity exponentially over time. From these observations it can be concluded that the immobilization of Alcalase strongly reduces the rate of hydrolysis of CalB by Alcalase.

The cause for the reduction in CalB hydrolysis by immobilization of Alcalase may have several reasons. The diameter of the pores of the beads onto which Alcalase is immobilized is approximately 70 nm and the approximate dimensions of CalB are 3 x 4 x 5 nm (Uppenberg *et al.* 1994). CalB, assuming it to be present as monomers, should thus be able to enter the pores of the beads. The time needed for CalB to diffuse into the center of the beads onto which Alcalase is immobilized can be roughly estimated using the average diameter of the beads (d) (2.25E-04 m) as

characteristic length, and 8E-11 $m^2\,s^{-1}$ as the diffusion coefficient of CalB (D) in solution, which can be estimated based on its molecular weight (Young *et al.* 1980). The Einstein approximation for the diffusion time (t_d),

$$t_d = \frac{d^2}{8D} \tag{2.3}$$

is 1.3 minutes. Not all the Alcalase will however be immobilized onto the internal surface of the beads. Some Alcalase will be immobilized onto the external surface of the beads, which leads to a smaller diffusion time. CalB also needs time to pass the stagnant layer surrounding the beads. This time can be calculated using the Sherwood number (Sh). If the Sherwood number is assumed to be equal to 2 because the beads are very small (Kragl *et al.* 1999), the time needed for CalB to pass the stagnant layer (t_s),

$$t_s = \frac{d^2}{6ShD} \tag{2.4}$$

is about 1 minute. The diffusion time and the time needed to pass the stagnant layer are very small compared with the 4 hours over which the loss activity of CalB is measured. Based on these calculations it is seems that CalB is not diffusion limited. However, the Einstein approximation for the diffusion time implies that molecules diffuse freely, which is likely not the case for native CalB inside the pores due to the possible tortuosity of the pores and due to a limitation in free moving space. The effective diffusion coefficient will be much smaller than in free diffusion, thereby leading to a significant increase in diffusion time and to a reduction in the rate of hydrolysis of CalB by Alcalase. Furthermore, the covalent immobilization of Alcalase probably inhibits the enzyme from peptide bond hydrolysis due to restrictions of its conformational flexibility or due to a suboptimal orientation.

If the inactivation rates of native and immobilized CalB by 1 mg of native Alcalase are compared using k of native CalB and k_2 of immobilized CalB, it can be concluded that native CalB is degraded more than 10^9x faster than immobilized CalB (Table 2.3). Only a fraction of 0.12 to 0.14 of immobilized CalB (x_1) inactivates at a high rate with constant k_1 (k_1 = 2.2 – 4.1 h^{-1}). The difference between native and immobilized CalB in the presence of native Alcalase can also be clearly seen in

the figures (Figures 2.3, 2.4, and 2.5). Covalent and adsorptive immobilization of CalB thus also strongly reduces the rate of hydrolysis of CalB by Alcalase.

Again, the cause for the reduction in CalB hydrolysis by Alcalase may have several reasons. The diameter of the pores of the beads onto which CalB is covalently immobilized averages approximately 70 nm and the average diameter of the pores of the beads onto which CalB is adsorbed is 15 nm. The approximate dimensions of Alcalase are 5 x 6 x 8 nm (Schmitke *et al.* 1997). Alcalase, assuming it to be present as monomers, should thus be able to enter the pores of the beads onto which CalB is covalently immobilized. Nevertheless, for Alcalase to pass through a pore of 15 nm inside which one or more CalB molecules (3 x 4 x 5 nm (Uppenberg *et al.* 1994)) are adsorbed will be very difficult. This will hinder Alcalase from coming into contact with CalB. Based on free diffusion, the time needed for Alcalase to diffuse into the center of the beads onto which CalB is immobilized and the time needed for Alcalase to pass the stagnant layer surrounding the beads is again much less than the 4 hours over which the loss activity of CalB is measured. Nevertheless, the possible tortuosity of the pores, and the limitation in free moving space will lead to a significant increase in diffusion time and to a reduction in the rate of hydrolysis of CalB by Alcalase. Furthermore, immobilization of CalB may cause the enzyme molecules to orient in such a way that Alcalase cannot easily hydrolyze the peptide bonds. The beads onto which CalB is adsorbed are negatively charged (Soledad de Castro & Sinisterra Gago 1998). Alcalase is, however, positively charged at pH 7.5 (assay conditions) as it has an isoelectric point of 9.4 (Sigma-Aldrich 2010). The charge of the supports will, therefore, not lead to an increase in diffusion time.

The activity of especially covalently immobilized CalB decreases with an increasing amount of native Alcalase (Figure 2.4 and Table 2.3). The values of x_1, which is the fraction of the enzyme that inactivates at a high rate, increase with an increasing amount of native Alcalase. By increasing the amount of Alcalase, the driving force for Alcalase to enter the pores will be higher. Therefore, by increasing the amount of Alcalase, the rate of CalB inactivation also increases (*i.e.* x_1 increases). This would suggest that Alcalase is indeed diffusion limited. Nevertheless, it seems that after an initial fast rate of inactivation, the rate of inactivation almost stagnates,

which suggests a steady state has been reached. This apparent steady state is reinforced by the Michaelis-Menten correction of covalently immobilized CalB and native Alcalase (when using 10 mg of native Alcalase). If the lower boundaries of K_m are used for both immobilized CalB and native Alcalase, k_2 increases somewhat.

The disadvantage of non-covalent adsorption commonly mentioned in literature is the ease of leaching of the enzyme from the carrier in aqueous media. Leaching would lead to a fast degradation of CalB in the presence of Alcalase, if the immobilized CalB is assumed to be less accessible to native Alcalase than native CalB is to native Alcalase. In our system and within a time span of about 4 hours, leaching of CalB does not seem to occur significantly because the inactivation of adsorbed CalB in the presence of Alcalase is not faster than the inactivation of covalently immobilized CalB.

If the immobilization of Alcalase is compared with the immobilization of CalB with respect to the prevention of proteolysis, it can be concluded that a small fraction of 0.12 to 0.14 of immobilized CalB (x_1) in the presence of 1 mg of native Alcalase inactivates at a high rate with constant k_1 (k_1 = 2.2 – 4.1 h^{-1}) (Table 2.3). The rest of the immobilized CalB fraction inactivates at a minimal rate. Native CalB in the presence of immobilized Alcalase shows no significant decrease in activity for about 4 hours. It seems that immobilization of Alcalase is slightly more effective in minimizing hydrolysis of CalB by Alcalase.

Immobilization of either CalB or Alcalase onto macroporous acrylic beads is thus effective in minimizing hydrolysis of CalB by Alcalase. Likely this finding can be applied to other immobilization methods and supports where either one of the enzymes is sterically hindered from coming into contact with the other enzyme, is reduced in its flexibility, or is oriented in such a way that peptide hydrolysis is not favorable.

Immobilized CalB was also incubated with immobilized Alcalase. As expected, the activity profile of immobilized CalB with immobilized Alcalase was not significantly different from the activity profile of solely immobilized CalB, both for adsorbed and covalently immobilized CalB (results not shown).

A novel option for one-pot systems in which two or more enzymes are involved that catalyze sequential reaction steps, would be to immobilize the enzymes onto

the same carrier. This would minimize transport limitations of substrates because after a substrate-molecule is converted by one enzyme it only has to diffuse over a short distance to reach to the next enzyme. Whether immobilization of the enzymes is practical will depend on the application. Immobilization of the enzymes will likely increase their stability, facilitate the recovery from the reaction medium, and allow them to be reused. Nevertheless, immobilization leads to additional costs (*i.e.* costs for the immobilization procedure) and loss of activity during immobilization (Kragl *et al.* 1999), which should be compensated for by increased effectivity.

2.4 Conclusion

Immobilization of both or either CalB or Alcalase onto macroporous beads is effective in largely preventing hydrolysis of CalB by Alcalase.

CHAPTER 3

Selecting optimal conditions for Alcalase CLEA-OM for synthesis of dipeptides in organic media

Vossenberg P, Beeftink HH, Nuijens T, Cohen Stuart MA, Tramper J. 2012. Selecting optimal conditions for Alcalase CLEA-OM for synthesis of dipeptides in organic media. Journal of Molecular Catalysis B: Enzymatic 75:43-49.

Abstract

In protease-catalyzed peptide synthesis, the availability of water is essential, as a compromise must be made between on the one hand the overall enzymatic activity and, on the other hand, the rate of product synthesis. Water is essential for enzyme activity, but at the same time causes hydrolytic side reactions. We studied the coupling of phenylalanine amide and the carbamoylmethyl ester of N-protected phenylalanine in tetrahydrofuran catalyzed by Alcalase CLEA-OM at a range of water activity (a_w) values, including the coupling in the presence of molecular sieves (*i.e.* at very low a_w values). The hydrolytic side reaction (in the present system only the hydrolysis of substrate occurs) was found to dominate above an a_w value of about 0.2. To prevent hydrolysis, the presence of molecular sieves was found to be necessary.

Keywords: *protease, dipeptide synthesis, water activity, molecular sieves, solvent*

3.1 Introduction

Natural and non-natural peptides are important for pharmaceutical and food applications (Guzmán *et al.* 2007). These peptides may be synthesized enzymatically. A possible peptide synthesis route, which is investigated in the frame of the Dutch national research program "Integration of Biosynthesis and Organic Synthesis" (IBOS), starts with activating the C-terminus of an N-protected amino acid using a lipase. Subsequently, this activated amino acid is coupled with an amino amide to give a dipeptide using a protease (Figure 3.1, reaction 1) (Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011; Vossenberg *et al.* 2011). If 'free' water is present in the reaction medium, hydrolytic side reactions may proceed in addition to the coupling reaction (Figure 3.1, reaction 2 and 3). To prevent the spontaneous hydrolysis of the activated amino acid (Figure 3.1, reaction 2) as well as the enzyme-catalyzed hydrolysis of the activated amino acid, of the formed dipeptide (Figure 3.1, reaction 3), and of the protease itself (autolysis), the availability of water in the system should be minimized. To achieve this, the coupling reaction can be carried out in a neat organic solvent. One should expect, however, that the organic solvent should not be completely dry as enzymes need some water to maintain their catalytically active conformation (Gaertner & Puigserver 1989; Adlercreutz 1991; Bordusa 2002). In a certain range, the more water, the greater the enzymatic activity (Zaks & Klibanov 1988b). Nevertheless, in the case of peptide synthesis (or any other reaction in which water is a substrate in hydrolytic reactions, *e.g.* transesterification) a compromise, with respect to the amount of water in the system, must be made between the overall enzymatic activity and the rate of specifically the product synthesis (Partridge *et al.* 1996).

Thermodynamic water activity (a_w) is a measure of the availability of water in a system and reflects the extent of the interactions of water with other system components. It thus depends on the hydrophilic or hydrophobic nature of the solvent. To hydrophilic solvents a lot more water has to be added than to hydrophobic solvents to achieve the same a_w. The a_w value determines the hydration level of the enzyme and thereby also its activity (Halling 1992; Ljunger *et al.* 1994).

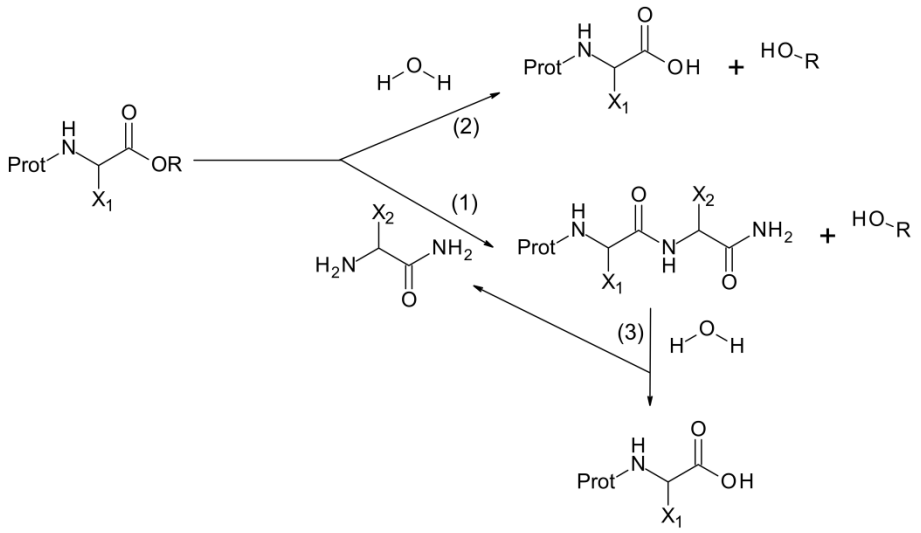

Figure 3.1. Coupling of an N-protected, C-terminally activated amino acid with an amino amide to give a dipeptide (1), hydrolysis of an N-protected, C-terminally activated amino acid (2), and hydrolysis of the formed dipeptide (3). X_1 and X_2 can be any amino acid side chain, but are in this study both a benzyl side chain. R is an activating group, which is in this study a carbamoylmethyl.

The present study focuses on the coupling of the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam), and phenylalanine amide (Phe-NH_2). A carbamoylmethyl ester was used instead of the more regularly used methyl or ethyl esters due to its positive effect on the coupling rate (Miyazawa *et al.* 2002). The coupling was catalyzed by Alcalase CLEA-OM in tetrahydrofuran (THF) at different a_w values. Protease-catalyzed peptide synthesis in monophasic organic solvents has been studied previously (Oyama *et al.* 1981; Ferjancic *et al.* 1990; Filippova & Lysogorskaya 2003; Belyaeva *et al.* 2005; Nuijens *et al.* 2011), including studies investigating the effect of water concentration on peptide synthesis catalyzed by different proteases: chymotrypsin (Gaertner & Puigserver 1989; Clapés *et al.* 1990; Kimura *et al.* 1990; Kise & Hayakawa 1991; Clapés *et al.* 1992; Jönsson *et al.* 1995; Capellas *et al.* 1996; Sergeeva *et al.* 1997), subtilisin Carlsberg (Sergeeva *et al.* 1997; Klein *et al.* 2000; Okazaki *et al.* 2000; Miyazawa *et al.* 2002), subtilisin BPN' (Kise & Hayakawa 1991), Alcalase (Chen *et al.* 1992; Hou *et al.* 2006; Li *et al.* 2008), and papain (Zhang *et al.* 1996). Nevertheless, the present contribution is to our

knowledge the first to investigate the coupling of Z-Phe-OCam and Phe-NH_2 in THF using Alcalase CLEA-OM at a range of a_w values, including the coupling in the presence of molecular sieves (*i.e.* at very low a_w values).

The aim of our study was to investigate up to which a_w value the rate of peptide synthesis could be increased without significantly increasing the rate of hydrolysis, *i.e.* without significantly decreasing the synthesis / hydrolysis (S/H) ratio. The a_w was varied from about 0 to 0.95 (in THF these a_w values correspond to 0 - 11 % (v/v) water). In addition, the pH of the buffer used to wash Alcalase CLEA-OM was varied from 7 to 11 to examine whether this had an effect on subsequent coupling in near-dry conditions. Furthermore, the pH of the buffer that was added directly to the reaction medium, to achieve an a_w of 0.6, was varied to observe whether this had an effect on the synthetic and hydrolytic reactions that take place at this a_w. The effect of the concentration of molecular sieves present in the reaction medium was studied in order to study the dipeptide synthesis at a_w values near 0.

3.2 Materials and Methods

3.2.1 Enzymes

Alcalase® (protease from *Bacillus licheniformis*) CLEA-OM® (formulation optimized for use in organic media) was obtained from CLEA Technologies (Delft, The Netherlands). Alcalase CLEA-OM contains the enzyme Alcalase from Novozyme Corporation (Bagsvaerd, Denmark).

3.2.2 Chemicals

All chemicals used were reagent or analytical grades. *tert*-Butanol (*t*-BuOH) and THF were dried over 3 Å molecular sieves, 8 - 12 mesh beads (Sigma- Aldrich, Zwijndrecht, The Netherlands), for at least 1 day prior to use. *t*-BuOH was pre-heated to a liquid (40 °C) prior to use. The N-protected Cam-ester of phenylalanine (Z-Phe-OCam) and N-protected phenylalanyl-phenylalanine amide (Z-Phe-Phe-NH_2) were a kind gift from DSM (Geleen, The Netherlands). Phenylalanine amide (Phe-NH_2), N-protected phenylalanine (Z-Phe-OH), and N-protected phenylalanyl-phenylalanine (Z-Phe-Phe-OH) were purchased from Bachem (Weil am Rhein, Germany).

3.2.3 Setting water activity

The a_w was set by adding an appropriate amount of water to the THF, to which the enzyme and substrates were added. The amount of water that needed to be added to achieve a certain a_w was calculated using the activity coefficient (γ_w) and the mole fraction of water (x_w),

$$a_w = \gamma_w x_w \tag{3.1}$$

γ_w can be calculated from (Gothard *et al.* 1976; Gmehling *et al.* 1977; Bell *et al.* 1997):

$$\ln(\gamma_w) = -\ln(x_w + \Lambda_{ws}(1-x_w)) + (1-x_w)\left(\frac{\Lambda_{ws}}{x_w + \Lambda_{ws}(1-x_w)} - \frac{\Lambda_{sw}}{\Lambda_{sw}x_w + (1-x_w)}\right) \tag{3.2}$$

where

$$\Lambda_{ws} = \frac{V_s}{V_w}\exp\left(\frac{-(\lambda_{ws} - \lambda_{ww})}{RT}\right) \tag{3.3}$$

and

$$\Lambda_{sw} = \frac{V_w}{V_s}\exp\left(\frac{-(\lambda_{sw} - \lambda_{ss})}{RT}\right) \tag{3.4}$$

where V_s and V_w are the molar volumes of the organic solvent, in this case THF and water, respectively. V_s is 81.55 ml mol^{-1} at 25 °C and V_w 18.07 ml mol^{-1} (Bell *et al.* 1997). For a THF-water mixture the value of the λ_{ws}-λ_{ww} Wilson coefficient is 7603 J mol^{-1} and the value of the λ_{sw}-λ_{ss} Wilson coefficient is 11212 J mol^{-1} (Bell *et al.* 1997). R is the gas constant (8.314 J mol^{-1} K^{-1}) and T the absolute temperature (K).

The above calculation is based on a binary system, in this case water and THF. For the enzymatic peptide synthesis the system contained additional compounds such as substrates, an enzyme formulation, and, in time, products. As the concentration of substrates (53 mM Phe-NH_2 and 37 mM Z-Phe-OCam) and the amount of enzyme added to the reaction mixture (6 mg/ml) is rather low, we assume the a_w of our system to be similar to the calculated a_w based on a binary system.

The water content of the THF solutions was measured by Karl-Fischer titration (Mettler Toledo DL38; Tiel, The Netherlands) after the incubation period in which

dipeptide synthesis could take place. Based on this measured water content, the a_w of the reaction mixtures was recalculated using the equations above.

We chose to set the a_w by adding an appropriate amount of water to the THF instead of equilibrating with saturated salt solutions or salt hydrate pairs (Halling 1994). When THF was equilibrated with a saturated salt solution for 24 hours, a lot of THF evaporated and was found in the salt solution. When solvents dissolute in the salt solution it can alter the a_w of the salt solution (Hutcheon *et al.* 1997). In addition, we did not want to add the salt hydrate pairs directly to the system containing the enzyme because the salt hydrates may not be compatible with the enzyme (Hutcheon *et al.* 1997) and the salt hydrates may have acid-base effects on the enzyme (Fontes *et al.* 2003).

3.2.4 Enzymatic peptide synthesis

Enzymatic reactions were carried out at 25 °C in 1.5 ml glass vials placed on a blood rotator spinning at 15 rpm. Alcalase CLEA-OM was washed successively with 1 ml of anhydrous *t*-BuOH and 1 ml of THF to remove residual water. This washing step involved adding the solvent to the Alcalase formulation, shaking the sample, allowing the sample to settle, and removing the solvent using a pipette. This method was analogous to the procedure used to produce propanol-rinsed enzyme preparations (Partridge *et al.* 1998; Moore *et al.* 2001).

For some experiments, Alcalase CLEA-OM was washed with 1 ml of Milli-Q or buffer prior to washing with *t*-BuOH and THF.

In an attempt to achieve a completely dry Alcalase CLEA-OM formulation, 75 mg 3 Å molecular sieves, 8 - 12 mesh beads, were added to 1.7 mg of solid Alcalase CLEA-OM particles. The mixture was stored at 4 °C for 18 hours prior to use.

In order to dry Alcalase CLEA-OM in the presence of THF, different amounts of 3 Å molecular sieve powder (Sigma-Aldrich) were added to the enzyme preparation and stored in THF at 25 °C for 24 hours prior to use.

For the enzymatic peptide synthesis, 150 µl THF containing 16 µmol Phe-NH_2 and 150 µl THF containing 11 µmol Z-Phe-OCam were added to 1.7 mg of the enzyme. To set the a_w of the reaction mixture, either molecular sieves or an appropriate

amount of water were added. Samples (50 µl) were diluted with 700 µl dimethyl sulfoxide (DMSO) before HPLC analysis.

Except for the reactions used to investigate dipeptide synthesis at a_w values near 0, 75 mg 3 Å molecular sieves, 8 - 12 mesh beads, were used. For the reactions used to investigate dipeptide synthesis at a_w values near 0, different amounts of 3 Å molecular sieve powder were added to the reaction mixture instead of the coarse beads. Due to the small volumes used in our system, the amount and therefore the adsorptive capacity of powder can be controlled better (by weighing off a specific amount of powder) than that of beads. We assume the molecular sieve powder to have the same adsorptive capacity for water molecules as the molecular sieve beads because, similarly to the beads, it was sold as a drying agent. Furthermore, the powder has the same elemental composition as the beads.

To study the effect of pH on dipeptide synthesis, the following buffers were used: 50 mM Tris (hydroxylmethyl) aminomethane – hydrochloric acid buffer (pH 7 - 9), 50 mM sodium carbonate – sodium bicarbonate buffer (pH 9.5 -10.5), and 50 mM sodium bicarbonate buffer – sodium hydroxide (pH 11).

3.2.5 Substrate and dipeptide (product) hydrolysis

To investigate whether hydrolysis of the substrate, Z-Phe-OCam, takes place in our system, 300 µl THF containing 11 µmol Z-Phe-OCam was incubated at 25 °C with 1.7 mg of Alcalase CLEA-OM, that was not washed with anhydrous *t*-BuOH and THF prior to use. A sample, for HPLC analysis, was taken after 24 hours of incubation time.

To test spontaneous substrate hydrolysis, 150 µl THF containing 15 µmol Phe-NH_2 and 150 µl THF containing 11 µmol Z-Phe-OCam were incubated at 25 °C without enzyme at a_w values ranging from 0.4 to 0.95. Samples, for HPLC analysis, were taken after 24 hours, and after 6 or 8 days of incubation time.

To investigate whether hydrolysis of the dipeptide, Z-Phe-Phe-NH_2, takes place in our system, Z-Phe-Phe-OH was incubated at 25 °C with 1.7 mg of Alcalase CLEA-OM, that was washed successively with 1 ml of anhydrous *t*-BuOH and 1 ml of THF prior to use, at a range of a_w values (a_w = 0 - 0.95). The possible hydrolysis

of Z-Phe-Phe-OH was assumed to be similar to Z-Phe-Phe-NH_2. Samples, for HPLC analysis, were taken after 24 hours and 7 days of incubation time.

3.2.6 HPLC analysis

The amounts of dipeptide (Z-Phe-Phe-NH_2), Cam-ester (Z-Phe-OCam), and N-protected phenylalanine (Z-Phe-OH) were analyzed by HPLC (Thermo Separation Products P4000 pump and AS3000 autosampler) using a reversed-phase column (Inertsil ODS-3, C18, 5 µm, 150 × 4.6 mm) at 40 °C. UV detection was done at 220 nm using an Ultimate 3000 Diode Array Detector (Dionex). The gradient program was: 0 - 25 min linear gradient ramp from 5 % to 98 % eluent B, 25 - 29 min linear gradient ramp back to 5 % eluent B, 29 - 40 min 5 % eluent B (eluent A: 0.5 ml l^{-1} methane sulfonic acid (MSA) in Milli-Q, eluent B: 0.5 ml l^{-1} MSA in acetonitrile). The flow was 1 ml min^{-1}. Injection volumes were 20 µl. Quantitative analysis was carried out using calibration curves of Z-Phe-Phe-NH_2, Z-Phe-OCam, and Z-Phe-OH.

HPLC analysis was not a problem for the system in which CLEA-OM and molecular sieve beads, or CLEA-OM and molecular sieve powder were used, as enough reaction liquid remained for the analysis.

3.3 Results and discussion

3.3.1 Substrate and dipeptide (product) hydrolysis

To investigate whether hydrolysis of the substrate, Z-Phe-OCam (Figure 3.1, reaction 2), takes place in our system, Z-Phe-OCam was incubated with Alcalase CLEA-OM, that was not washed with anhydrous *t*-BuOH and THF prior to use. After 24 hours of incubation in the absence of both molecular sieves and additional water, Z-Phe-OH was detected. The original Alcalase CLEA-OM formulation thus contains enough water for hydrolysis of Z-Phe-OCam to take place.

Z-Phe-OCam was also incubated without enzyme at a_w values ranging from 0.4 to 0.95, to test spontaneous hydrolysis. At a_w values ranging from 0.4 to 0.8 no Z-Phe-OH was detected after 6 days of incubation. At an a_w value of 0.95, 5 % of the Z-Phe-OCam was hydrolyzed after 24 hours and 35 % after 8 days of incubation. So

only above an a_w value of minimally 0.8 some measurable spontaneous hydrolysis of the substrate occurs.

To investigate whether hydrolysis of the dipeptide, Z-Phe-Phe-NH_2 (Figure 3.1, reaction 3), takes place in our system, Z-Phe-Phe-OH was incubated with Alcalase CLEA-OM, that was washed successively with anhydrous *t*-BuOH and THF prior to use, at a range of a_w values (a_w = 0 - 0.95). The possible hydrolysis of Z-Phe-Phe-OH was assumed to be similar to Z-Phe-Phe-NH_2. After 7 days of incubation, no Z-Phe-OH was detected. Also in later reactions in which Z-Phe-Phe-NH_2 was formed, no product hydrolysis was apparent in time. Hydrolysis of the dipeptide (Figure 3.1, reaction 3) thus does not take place in our system. This is in line with the work from Li *et al*. in which no hydrolysis of Z-Asp-Val-NH_2 was observed by native Alcalase in acetonitrile containing 10 % buffer (Li *et al.* 2008). Also Hou *et al.* did not observe hydrolysis of Bz-Arg-Gly-NH_2 by Alcalase in acetonitrile containing 10 % buffer (Hou *et al.* 2006). Although Jönsson *et al*. did observe hydrolysis of Ac-Phe-Ala-NH_2 (by immobilized α-chymotrypsin in acetonitrile containing 10 % water), the substrate ester, Ac-Phe-OEt, was a considerably better substrate for hydrolysis than the dipeptide (Jönsson *et al.* 1995).

Apparently, Z-Phe-OCam is a much better substrate in the hydrolytic reaction for the enzyme than the current dipeptide; Z-Phe-Phe-NH_2 hydrolysis can thus be neglected. In the present system the incubation time is thus not a critical parameter in the optimization of the dipeptide synthesis because once the dipeptide is formed, it will not be hydrolyzed.

3.3.2 Effect of a_w on dipeptide synthesis

The coupling of Z-Phe-OCam and Phe-NH_2, catalyzed by Alcalase CLEA-OM, was investigated at a range of a_w values. The enzyme formulation was washed successively with anhydrous *t*-BuOH and THF before the substrates, and molecular sieve beads or an appropriate amount of water were added. The enzyme and substrates were incubated for 24 hours. In the presence of molecular sieves the a_w of the reaction mixture can be safely assumed to be the lowest attained and approaching zero, *i.e* $a_w \approx 0$.

Figure 3.2 shows the effect of a_w on the total catalytic activity (synthesis plus hydrolysis). The a_w value on the x-axis is a recalculated a_w value according to the THF water content determined with Karl-Fischer titration after peptide synthesis. The relative conversions in Figure 3.2 (left y-axis) were calculated by normalization with respect to the conversion at a_w = 0.95 with CLEA-OM that was washed with anhydrous *t*-BuOH and THF, and immediately added to the reaction mixture. At an a_w value of 0.95, 6.6 mmol Z-Phe-OCam was converted per gram of CLEA-OM in 24 hours. Next to achieving a high catalytic activity at high a_w values, a high catalytic activity was also achieved at very low a_w (a_w < 0.1) values. The amount of spontaneous hydrolysis of Z-Phe-OCam at high a_w (a_w > 0.8) values, mentioned above, was negligible compared to the total amount of hydrolysis at these a_w values.

Figure 3.2 also shows the effect of a_w on the S/H ratio. The hydrolysis reaction consists only of the hydrolysis of the activated amino acid (Figure 3.1, reaction 2). At extremely low a_w values, the S/H ratio is theoretically infinite ('inf') as no hydrolysis of the substrate was measured. The S/H ratio decreases rapidly with increasing a_w values. Based on the measured S/H ratio values at different a_w values, it can be concluded that from an a_w value of about 0.2, the S/H ratio becomes less than 1. The hydrolysis reaction thus dominates above an a_w value of about 0.2. At an a_w value of 0.95, only a minimal amount of dipeptide (Z-Phe-Phe-NH_2) was synthesized. The S/H ratio at this a_w is 0.01.

If the hydrolysis of the substrate should be minimal because the substrate is, for example, very expensive, the a_w should thus be low (a_w << 0.2). To prevent the formation of a hydrolysis product (Z-Phe-OH), the presence of molecular sieves was found to be necessary, *i.e.* the a_w must be extremely low to prevent hydrolysis. In the absence of both molecular sieves and additional water, hydrolysis was still observed. This means that there is still enough water left on the enzyme formulation that can compete with the dipeptide synthesis reaction. The successive washing steps with anhydrous *t*-BuOH and THF thus do not remove all the water from the enzyme formulation. The loss of water from the enzyme formulation to the bulk solvent means that the two pertinent phases (*i.e.* the THF and the enzyme formulation) were not at equilibrium. The a_w of the bulk solvent

was set by the addition of an appropriate amount of water to the THF. During the 24 hour incubation period for peptide synthesis, the water was then likely redistributing between the CLEA-OM and the bulk solvent.

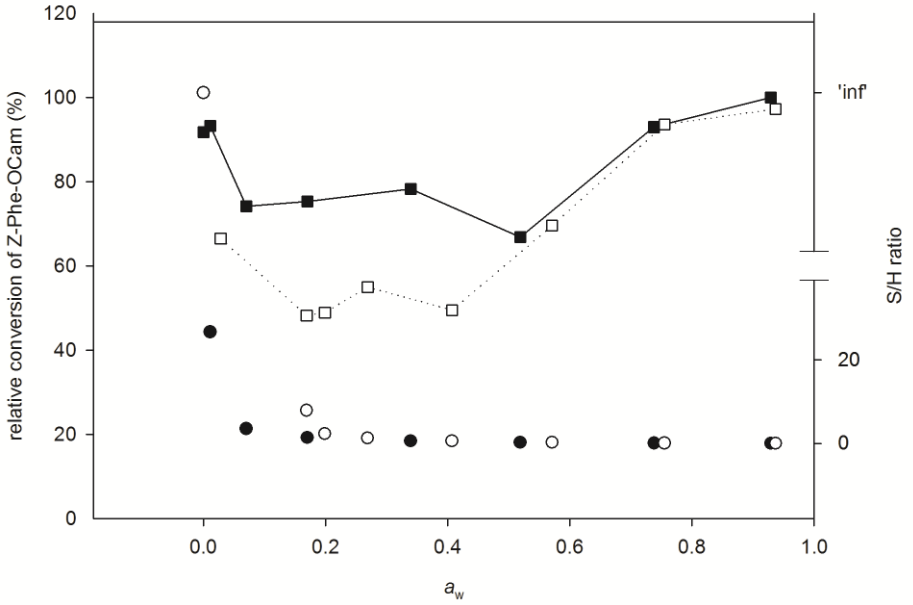

Figure 3.2. Effect of a_w on the total catalytic activity (■) and on the synthesis/hydrolysis (S/H) ratio (●) of Alcalase CLEA-OM immediately, after washing with anhydrous *t*-BuOH and THF, added to the reaction mixture, and on the total catalytic activity (□) and on the S/H ratio (○) of Alcalase CLEA-OM equilibrated (24 h) before addition to the reaction mixture. The total conversion of Z-Phe-OCam at a certain a_w is relative to the highest conversion obtained.

In order to compare the above results with a system in which CLEA-OM is equilibrated to the same a_w as the bulk solvent, the experimental set-up was modified. Before incubating the enzyme and substrates for 24 hours, CLEA-OM was incubated with 1.5 ml of THF at a range of a_w values. The water transferred from or to the enzyme was so minimal that it did not measurably change the a_w of the bulk THF. After 24 hours, the THF was removed and THF containing the substrates was added. The a_w of the THF containing the substrates was set 4 hours before incubation with the enzyme. As was observed in the system in which CLEA-OM was used immediately, the highest catalytic activity was achieved at the highest a_w value tested (0.95) (Figure 3.2). Nevertheless, in comparison to the system in

which CLEA-OM was used immediately, a lower catalytic activity was achieved at a_w values between 0 and 0.4. The relative rate remained approximately constant at 52 % in the a_w range of 0.2 to 0.4. Above an a_w value of 0.4, the relative rate increased with a_w. As was observed in the system in which CLEA-OM was used immediately, the S/H ratio in the equilibrated system also decreased rapidly with increasing a_w values and the hydrolysis reaction remained to dominate above an a_w value of about 0.2 (Figure 3.2).

The main difference between the system in which CLEA-OM was used immediately and the equilibrated system, is the relatively high catalytic activities observed in the system in which CLEA-OM was used immediately at the lower a_w ($a_w < 0.4$) values. These high enzymatic activities, as compared to those observed with the equilibrated system, indicate that it might be quite interesting to use this 'hydration gradient' (and therefore non-equilibrium situation) between the enzyme formulation and the bulk solvent in dipeptide synthesis. Indeed, initially relatively high catalytic activities and high S/H ratios can be achieved using a relatively dry bulk solvent and a hydrated enzyme formulation. In line with this thought, Basso *et al.* developed an innovative method to maintain the hydration state of the enzyme while preventing hydrolytic side reactions. They adsorbed hydrated thermolysin onto Celite rods and carried out an enzymatic dipeptide synthesis in toluene at an a_w value of 0.7 - 0.75 (Basso *et al.* 2000).

3.3.3 Effect of pH on dipeptide synthesis

The pH of the buffer used to wash Alcalase CLEA-OM was varied from 7 to 11 to examine whether this had an effect on the subsequent coupling in near-dry conditions (Figure 3.3). The following buffers were used: 50 mM Tris (hydroxylmethyl) aminomethane – hydrochloric acid buffer (pH 7 - 9), 50 mM sodium carbonate – sodium bicarbonate buffer (pH 9.5 - 10.5), and 50 mM sodium bicarbonate buffer – sodium hydroxide (pH 11). Miyazawa *et al.* observed a large effect of the pH of the buffer solution from which lyophilized enzymes were prepared on the peptide yield, with an optimum at pH 10.7 (Miyazawa *et al.* 2002). In addition, Li *et al.* observed a significant effect of the pH of the 10 % (v/v) buffer

solution that was added to acetonitrile on the peptide yield, with an optimum at pH 10 (Li *et al.* 2008).

Alcalase CLEA-OM was hydrated with buffer and subsequently washed with anhydrous *t*-BuOH and THF. The enzyme and substrates were incubated for 24 hours in the presence of molecular sieve beads.

The y-axis of Figure 3.3 (as well as the y-axis of Figure 3.4 and Figure 3.5) is labeled 'relative composition.' The relative composition of, for example, Z-Phe-Phe-NH_2 was calculated as follows:

$$relative\ composition\ ZPhePheNH_2(\%) = \frac{[ZPhePheNH_2]_t}{[ZPheOCam]_0} \times 100\%$$

where $[ZPhePheNH_2]_t$ is the concentration of Z-Phe-Phe-NH_2 at the time of measurement and $[ZPheOCam]_0$ is the initial concentration of Z-Phe-OCam, both in mol ml^{-1}.

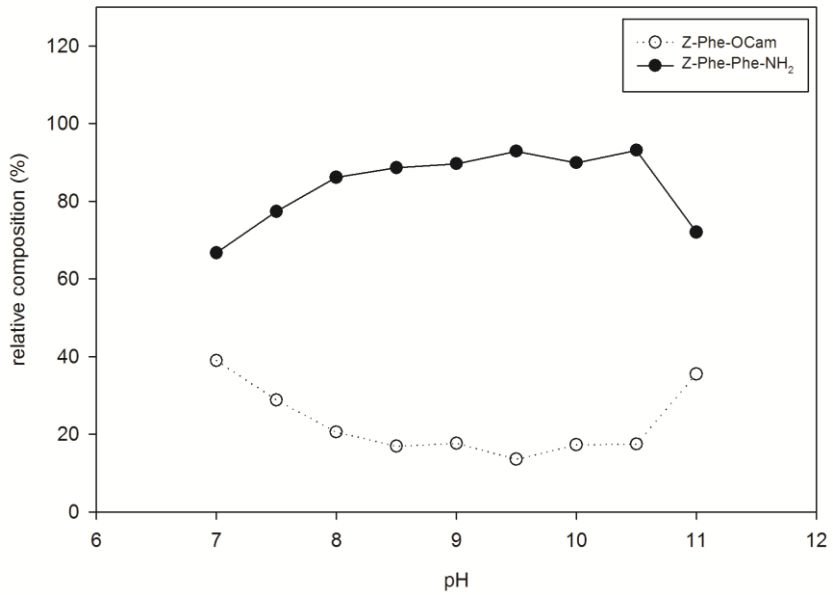

Figure 3.3. Effect of the pH of the washing buffer solution on the relative composition of the reaction mixture after 24 hours of incubation at 25 °C using Alcalase CLEA-OM in near-dry conditions.

The pH of the buffer used to wash Alcalase CLEA-OM did have an effect on the subsequent coupling (Figure 3.3). A broad pH optimum exists ranging from pH 8.0

to 10.5. Likely the small amounts of buffer that were present in the reaction mixture and surrounding the enzyme have an effect on the synthetic activity of the enzyme. These residual amounts of buffer were, however, so small that no hydrolysis was observed. This enhancement in synthetic activity between pH 8.0 and 10.5 coincides partly with the pH optimum of Alcalase activity in aqueous conditions, which is between pH 8 and 9 (Uhlig 1998). In addition, only deprotonated Phe-NH_2 can react to form the dipeptide and this form of Phe-NH_2 is available at alkaline pH (Bordusa 2002; Salam *et al.* 2008).

In order to investigate whether a pH optimum also exists when buffer is added directly to the reaction medium, the effect of the buffer pH on the reactions catalyzed by Alcalase CLEA-OM at an a_w of 0.6 was studied (Figure 3.4). At an a_w of 0.6 both synthesis and hydrolysis reactions occur. Alcalase CLEA-OM was hydrated with buffer, that had the same pH and concentration as the buffer added to the reaction mixture, and subsequently washed with anhydrous *t*-BuOH and THF. The same buffers were used as above, when studying the effect of the washing buffer pH on the subsequent coupling in near-dry conditions. The enzyme and substrates were incubated for 24 hours at an a_w of 0.6. To achieve an a_w of 0.6, 5.9 µl of buffer was added to 300 µl of THF containing 1.7 mg CLEA-OM.

In a monophasic organic solvent, pH is undefined (Straathof 2006). The enzyme is a polyelectrolyte with a certain ionization state. The addition of 5.9 µl buffer to the reaction mixture (to achieve an a_w of 0.6) means that about 35 µg of buffer salts were added. It is doubtful whether this small amount of buffer salts can alter the ionization state of 1.7 mg CLEA-OM. The washing step with 1 ml of buffer before incubation, however, may have a more significant effect on the ionization state of the enzyme and therefore on the synthesis and hydrolysis reactions that occur (as was seen when studying the effect of the washing buffer pH on the subsequent coupling in near-dry conditions), as the volume used is much larger.

The rates of dipeptide synthesis and substrate hydrolysis do not seem to be significantly affected by the pH of the buffer solution that was added to the reaction mixture (Figure 3.4). No optimum was observed. The pH of the buffer at an a_w of 0.6 neither affects the synthetic nor the hydrolytic activity of the enzymes significantly at the optimum pH range.

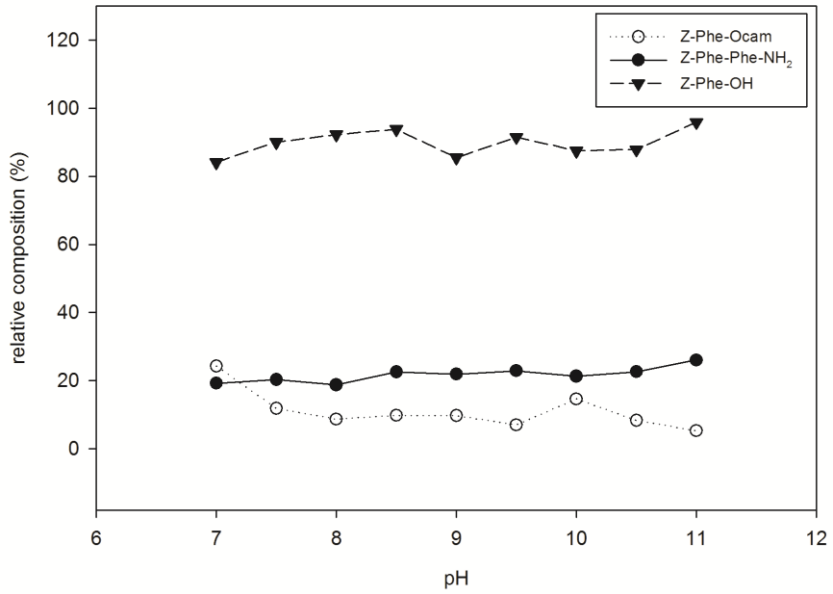

Figure 3.4. Effect of the pH of the buffer solution that was added to achieve an a_w of 0.6 on the relative composition of the reaction mixture after 24 hours of incubation at 25 °C using Alcalase CLEA-OM.

3.3.4 Dipeptide synthesis at very low a_w values

As stated before, the a_w of the reaction system must be extremely low to prevent hydrolysis. Nevertheless, the system should not be completely dry as enzymes need some water to maintain their catalytically active conformation (Gaertner & Puigserver 1989; Adlercreutz 1991; Bordusa 2002). We believe that an optimum in solely dipeptide synthesis can be achieved when the dipeptide synthesis is carried out near an a_w value of the bulk solvent of 0. In order to achieve a_w values near 0, molecular sieve powder, of which the concentration was varied, instead of molecular sieve beads were used. Due to the small volumes used in our system, the amount and therefore the adsorptive capacity of powder can be controlled better (by weighing off a specific amount of powder) than that of beads.

The effect of the molecular sieves concentration present in the reaction medium on the coupling of Z-Phe-OCam and Phe-NH_2 was investigated (Figure 3.5). Alcalase CLEA-OM was first hydrated with Milli-Q and subsequently washed with

anhydrous *t*-BuOH and THF. The enzyme and substrates were incubated for 3 hours in the presence of different amounts of molecular sieve powder.

With Karl Fischer titration it was not possible to accurately determine the water content of these samples as the water content is too low. Therefore, the specific a_w of the reaction systems containing different amounts of molecular sieve powder could not be calculated.

An optimum in dipeptide synthesis was achieved when the dipeptide synthesis was carried out in the presence of about 7.5 - 20 mg/ml of molecular sieve powder (Figure 3.5). When only a small amount of molecular sieve powder (< 3 mg/ml) was added to the reaction mixture hydrolysis could not be prevented. When a relatively large amount of molecular sieve powder was added to the reaction mixture (> 35 mg/ml) the rate of dipeptide synthesis was significantly reduced. Apparently, at this point, the activity of Alcalase CLEA-OM was reduced due to dehydration.

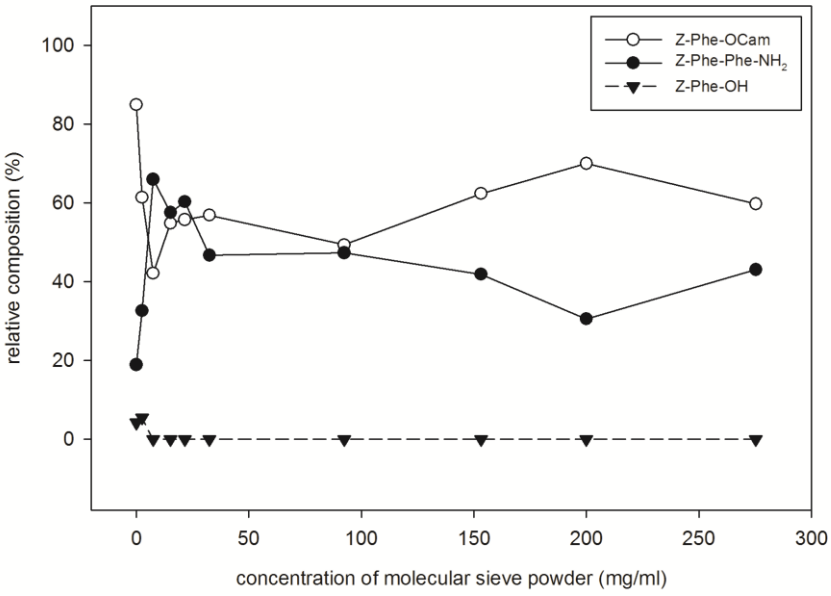

Figure 3.5. Effect of the molecular sieve powder concentration on the relative composition of the reaction mixture after 3 hours of incubation at 25 °C using Alcalase CLEA-OM.

The above investigation of the effect of the molecular sieves concentration present in the reaction medium on the coupling of Z-Phe-OCam and Phe-NH_2 using Alcalase

CLEA-OM was carried out in triplicate. Figure 3.5 is an illustrative example of the three series done. The results all show the same trend.

If it is assumed that molecular sieves are a perfect sink or at least have a very high partition coefficient for water, the enzyme formulation and the solvent may lose water in time. This would lead to inactive enzyme formulations as enzymes are known to require some essential water to maintain their catalytic active conformation (Gaertner & Puigserver 1989; Adlercreutz 1991; Bordusa 2002). An attempt was made to dry the Alcalase CLEA-OM formulation even further than with anhydrous *t*-BuOH and THF alone by storing it with molecular sieve beads for 18 hours before use in an enzymatic reaction. Indeed, this extremely dry Alcalase CLEA-OM formulation was found totally incapable of dipeptide synthesis.

To have an indication whether the enzyme formulation loses water in time in the presence of THF and molecular sieves, and thereby inactivates, non-washed Alcalase CLEA-OM was incubated in THF with different amounts of molecular sieve powder for 24 h before adding the substrates. The Alcalase CLEA-OM pre-incubated with 6 - 17 mg/ml molecular sieve powder converted on average 2.4 % of the substrate to dipeptide and 0.9 % to Z-Phe-OH in 3 h. The Alcalase CLEA-OM pre-incubated with 28 - 635 mg/ml molecular sieve powder converted on average 1.6 % of the substrate to dipeptide in 3 h and no hydrolysis was observed. Pre-incubation with a small amount of molecular sieve powder thus caused a somewhat higher dipeptide synthesis rate but hydrolysis could not be prevented. In any case, the conversion achieved by the Alcalase CLEA-OM formulation pre-incubated with molecular sieve powder is significantly lower than the > 40 % conversion achieved by the Alcalase CLEA-OM formulation to which molecular sieve powder was added at the same time as the substrates (Figure 3.5). The pre-incubation with molecular sieve powder thus seems to have dried the enzyme to such an extent that only a minimal activity remained. This result suggests that although the rate of catalysis in the systems to which molecular sieves are added at the same time as the substrates is very high in the first hours of incubation, due to the initially hydrated and thus active enzyme, the rate of catalysis will be minimal after 24 h of incubation, due to the significant

dehydration of the enzyme by the molecular sieves. The exact rate of this dehydration and its effect on the enzyme activity are subject to further study.

3.4 Conclusions

In the present system the incubation time is not a critical parameter in the optimization of the dipeptide synthesis because once the dipeptide is formed, it will not be hydrolyzed. Only hydrolysis of the substrate, Z-Phe-OCam, takes place.

The rate of peptide synthesis could not be increased by increasing a_w values without significantly increasing the rate of hydrolysis, *i.e.* without significantly decreasing the synthesis/hydrolysis (S/H) ratio. At extremely low a_w values, *i.e.* in the presence of an excess amount of molecular sieves, the S/H ratio is theoretically infinite as no hydrolysis product was measured. Above an a_w value of about 0.2, the hydrolysis reaction dominates.

The pH of the buffer used to wash Alcalase CLEA-OM has an effect on the coupling. A broad pH optimum exists ranging from pH 8.0 to 10.5. The pH of the buffer that is added directly to the reaction medium, to achieve an a_w of 0.6, neither affects the synthetic nor the hydrolytic activity of the enzyme.

An appropriate amount of molecular sieves can prevent hydrolysis of the substrate and still allow enzymatic activity. Nevertheless, if Alcalase CLEA-OM is pre-incubated with molecular sieves for 24 h before adding substrates, minimal activity is observed. The pre-incubation with molecular sieve powder thus seems to have dried the enzyme to such an extent that it had a negative effect on its activity. The use of molecular sieves over longer periods of time should therefore be carefully considered as they may dehydrate and thereby inactivate the enzyme in time.

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CHAPTER 4

Performance of Alcalase formulations in near-dry organic media: Effect of enzyme hydration on dipeptide synthesis

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Abstract

The use of different Alcalase formulations for protease-catalyzed dipeptide synthesis was investigated by studying the coupling of phenylalanine amide with the carbamoylmethyl ester of N-protected phenylalanine in tetrahydrofuran in the presence of molecular sieves (*i.e.* under near-dry conditions). Hydration prior to drying (with anhydrous *tert*-butanol and anhydrous tetrahydrofuran) of the Alcalase formulations resulted in a significant increase in the rate of the subsequent dipeptide synthesis. Repeated use, in the presence of molecular sieves, without intermediate rehydration led to inactivation of the enzyme. For three enzyme formulations this inactivation could be counteracted by intermediate rehydration. Inactivation of another enzyme formulation was only partially reversible by hydration. Alcalase immobilized onto dicalite with glutaraldehyde was found to be the most active in dipeptide synthesis, *i.e.* the formulation that initially produces the largest amount of product per gram of total formulation per unit of time. Due to its small particle size and its lack of operational stability, this formulation may nevertheless not be the best choice for the synthesis of dipeptides in neat organic media on a large scale. The most promising enzyme formulation for this is Alcalase covalently immobilized onto macroporous acrylic beads due to its reasonable activity, its seemingly good operational stability, and its practical size and uniform shape.

Keywords: *immobilized protease, peptide synthesis, molecular sieves, near-anhydrous*

4.1 Introduction

Natural and non-natural peptides play an important role in the fields of health care, nutrition, and cosmetics (Vulfson 1993; Lintner & Peschard 2000; Guzmán *et al.* 2007; Vivó-Sesé & Pla 2007; Stevenson 2009). These peptides may be synthesized chemo-enzymatically for instance by protease-catalyzed coupling of an amino amide and a chemically synthesized C-terminally activated N-protected amino acid, *e.g.* an N-protected carbamoylmethyl (Cam) ester (Miyazawa *et al.* 2002; Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011; Vossenberg *et al.* 2011; Vossenberg *et al.* 2012a). To prevent hydrolytic side reactions (hydrolysis of the activated amino acid, of the formed dipeptide, and of the protease itself), the availability of water in the system should be minimized, *e.g.* by executing the enzymatic peptide synthesis step in a neat organic solvent (Ljunger *et al.* 1994; Klibanov 2001). The system (reaction mixture and enzyme) should, however, not be completely dry as enzymes need some water to maintain their catalytically active conformation (Zaks & Klibanov 1988a; Zaks & Klibanov 1988b; Gaertner & Puigserver 1989; Adlercreutz 1991; Volkin *et al.* 1991; Ljunger *et al.* 1994; Klibanov 2001; Bordusa 2002; Halling 2004; Paravidino *et al.* 2010; Vossenberg *et al.* 2012a).

The present study focuses on the coupling of the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam) and phenylalanine amide (Phe-NH_2) (Figure 4.1). A carbamoylmethyl ester was used instead of the more regularly used methyl or ethyl esters because of its positive effect on the coupling rate (Miyazawa *et al.* 2002). The coupling was executed with different commercially available Alcalase formulations (Alcalase is also referred to as subtilisin A or subtilisin Carlsberg (De Lange & Smith 1968; Ottesen & Svendsen 1970; Gupta *et al.* 2002; Sigma-Aldrich 2010)) in tetrahydrofuran (THF) in the presence of molecular sieves (*i.e.* under near-dry conditions at low water activity), which were assumed to be stable in the process. In previous work we found that the rate of dipeptide synthesis could not be increased by increasing water activity values without significantly increasing the rate of hydrolysis, *i.e.* without significantly decreasing the synthesis / hydrolysis

(S/H) ratio (Vossenberg *et al.* 2012a). An appropriate amount of molecular sieves effectively prevents hydrolysis but still allows enzymatic activity (Vossenberg *et al.* 2012a).

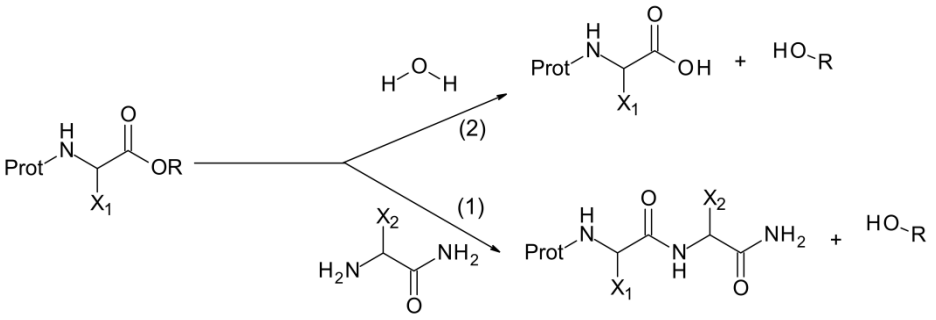

Figure 4.1. Coupling of an N-protected, C-terminally activated amino acid with an amino amide to give a dipeptide (1), and hydrolysis of an N-protected, C-terminally activated amino acid (2). X_1 and X_2 can be any amino acid side chain, but are in this study both a benzyl side chain. R is an activating group, which is in this study a carbamoylmethyl.

Protease-catalyzed peptide synthesis in monophasic organic solvents has been studied previously, with different proteases: chymotrypsin (Gaertner & Puigserver 1989; Clapés *et al.* 1990; Kimura *et al.* 1990; Kise & Hayakawa 1991; Clapés *et al.* 1992; Richards *et al.* 1993; Jönsson *et al.* 1995; Capellas *et al.* 1996; Sergeeva *et al.* 1997), subtilisin Carlsberg (Ferjancic *et al.* 1990; Sergeeva *et al.* 1997; Klein *et al.* 2000; Okazaki *et al.* 2000; Miyazawa *et al.* 2002), subtilisin BPN' (Kise & Hayakawa 1991), subtilisin 72 (Belyaeva *et al.* 2005), thermolysin (Oyama *et al.* 1981; Kimura *et al.* 1990; Nakanishi *et al.* 1990; Filippova & Lysogorskaya 2003), Alcalase (Chen *et al.* 1992; Hou *et al.* 2006; Li *et al.* 2008; Nuijens *et al.* 2011), and papain (Zhang *et al.* 1996). Nevertheless, the present contribution is to our knowledge the first to investigate the coupling of Z-Phe-OCam and Phe-NH_2 in THF with different Alcalase formulations ('hydrated' and 'dry') in the presence of molecular sieves (*i.e.* under near-dry conditions at low water activity) and subsequently compare the formulations with respect to their initial peptide synthesis activity and their applicability on a large scale.

The aim of this study was twofold: (1) to investigate the effect of initial hydration state of selected Alcalase formulations, achieved by a well-defined pretreatment protocol, on the subsequent dipeptide synthesis rate, and (2) to compare these formulations with respect to their applicability in dipeptide synthesis.

To achieve these aims, several commercially available Alcalase formulations, both 'hydrated' and 'dry,' were applied in the coupling of Z-Phe-OCam and Phe-NH_2. In addition, the repeated use (with and without intermediate rehydration) and thereby the operational stability of the four most active Alcalase formulations were compared. For this, the conversion of Z-Phe-OCam to dipeptide was monitored in time in two subsequent batch reactions. Furthermore, Alcalase formulation properties (particle size and shape, enzyme leaching from the support, and cost of immobilization) that are valuable for the synthesis of dipeptides in organic media were evaluated.

4.2 Materials and Methods

4.2.1 Enzymes

The Alcalase formulations used in the present investigation and a description of the formulations are listed in Table 4.1. The formulations from CLEA Technologies (CLEA Technologies 2010), ChiralVision (ChiralVision 2010), and DSM contain Alcalase from Novozyme Corporation (Bagsvaerd, Denmark). The formulations from SPRIN Technologies contain subtilisin A from *Bacillus species.* In the formulation from ChiralVision, Alcalase is covalently immobilized onto Immobeads 150 (cross-linked copolymer of methacrylate carrying oxirane groups) produced by ChiralVision in cooperation with FermentaBiotech Ltd. (Maharashtra, India).

4.2.2 Chemicals

tert-Butanol (*t*-BuOH) and THF were dried over activated 3 Å molecular sieves, 8 - 12 mesh beads ($K_nNa_{12-n}[(AlO_2)_{12}(SiO_2)_{12}] \cdot xH_2O$, Sigma-Aldrich, 208582), prior to use. *t*-BuOH was pre-heated to a liquid (40 °C) prior to use. The N-protected Cam-ester of phenylalanine (Z-Phe-OCam) and N-protected phenylalanyl-phenylalanine amide (Z-Phe-Phe-NH_2) were a kind gift from DSM (Geleen, The Netherlands), and used as supplied. Phenylalanine amide (Phe-NH_2) and N-protected phenylalanine (Z-Phe-OH) were purchased from Bachem (Weil am Rhein, Germany). Deionized water (Milli-Q) was used throughout.

Table 4.1. Alcalase formulations: abbreviations as used in the present investigation, full name / description, particle diameter, and supplier.

Abbreviation	Full name / description	Particle diameter	Supplier
Native	lyophilized native protease from *Bacillus licheniformis*	Fine powder	Sigma-Aldrich (Zwijndrecht, The Netherlands)
Cov	Alcalase covalently immobilized onto macroporous acrylic beads (Immobeads 150)	150 – 300 μm (ChiralVision 2011)	ChiralVision (Leiden, The Netherlands)
C-OM	Cross-linked enzyme aggregates (CLEA) containing Alcalase, CLEA-OM, formulation optimized for use in organic media	1 μm, but forms clusters (Sheldon *et al.* 2005)	CLEA Technologies (Delft, The Netherlands)
C-tBu	Alcalase CLEA-tBu, formulation precipitated and cross-linked in *tert*-butanol	1 μm, but forms clusters (Sheldon *et al.* 2005)	CLEA Technologies (Delft, The Netherlands)
C-ST	Alcalase CLEA-ST, standard CLEA formulation	1 μm, but forms clusters (Sheldon *et al.* 2005)	CLEA Technologies (Delft, The Netherlands)
Dic	Alcalase adsorbed onto dicalite 478 and cross-linked with glutaraldehyde	8 μm (Lin'kova *et al.* 1976)	A kind gift from DSM (Geleen, The Netherlands)
S-epo	Alcalase covalently immobilized onto epoxy acrylic resin	100 – 300 μm (SPRIN Technologies 2011)	SPRIN Technologies (Trieste, Italy)
S-imi	Alcalase covalently immobilized onto amino acrylic resin	100 – 300 μm (SPRIN Technologies 2011)	SPRIN Technologies (Trieste, Italy)

4.2.3 Enzyme pretreatment

The Alcalase formulations were pretreated in two different ways (the amounts of enzyme used are specified below, in section '4.2.4 Enzymatic peptide synthesis (1-timepoint measurement to compare Alcalase formulations)'). 'Dry' enzyme was obtained by washing with successively 1 ml of anhydrous *t*-BuOH and 1 ml of anhydrous THF. 'Hydrated' enzyme was obtained by washing with successively 1 ml of Milli-Q, 1 ml of anhydrous *t*-BuOH, and 1 ml of anhydrous THF. This treatment is expected to hydrate the enzyme and to remove the excess water. A

washing step involved adding washing liquid (Milli-Q, *t*-BuOH, or THF) to the Alcalase formulation, shaking the sample for 10 seconds, centrifuging the sample for 2 minutes at 10,000 rpm in order to facilitate the separation of the washing liquid and the enzyme formulation, and removing the washing liquid with a pipette. This method was analogous to the procedure used to produce propanol-rinsed enzyme preparations (Partridge *et al.* 1998; Moore *et al.* 2001).

Native Alcalase was not pretreated when applied 'dry,' assuming the freeze-dried powder to be dry. When native Alcalase was applied 'hydrated', it was hydrated by water vapor through the gas phase: it was placed for 96 h at 4 °C in an exsiccator containing Milli-Q.

4.2.4 Enzymatic peptide synthesis (1-timepoint measurement to compare Alcalase formulations)

For the enzymatic peptide synthesis (1-timepoint measurement), 150 µl THF containing 5.9 µmol Phe-NH_2 and 150 µl THF containing 3.5 µmol Z-Phe-OCam was added to the enzyme formulation. The amount of enzyme formulation was chosen to reach approximately 50 % conversion of the Cam-ester in 1 hour, with a 'hydrated' enzyme formulation. The following amounts of enzyme formulation were used: 5.5 mg of native Alcalase, 3 mg of Cov, 2.6 mg of CLEA-OM, 2.6 mg of CLEA-tBu, 8.5 mg of CLEA-ST, 1 mg of Dic, 30 mg of S-epo, and 3 mg of S-imi. To obtain near-dry conditions during peptide synthesis, 75 mg of 3 Å molecular sieves, 8 - 12 mesh beads, were added. Samples (50 µl) were diluted with 700 µl dimethyl sulfoxide (DMSO) to stop the reaction before HPLC analysis. The peptide synthesis was repeated 3 times for each enzyme formulation and carried out at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 30 rpm.

The reaction was stopped after 1 hour of incubation except for the conversions catalyzed by 'dry' Cov, 'dry' C-OM, 'dry' S-epo, and native Alcalase. After 1 hour of incubation, these formulations only achieved minimal conversion. For practical reasons (preventing the use of large amounts of enzyme), these formulations were incubated for 24 h in order to achieve a better measurable conversion.

4.2.5 Enzymatic peptide synthesis (repeated use)

To investigate the repeated use of the Alcalase formulations, 900 µl THF containing 29.4 µmol Phe-NH_2 and 900 µl THF containing 18.0 µmol Z-Phe-OCam were added to a 'hydrated' enzyme formulation. The peptide synthesis was carried out at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 30 rpm. The enzyme concentrations were identical to the concentrations for the 1-timepoint measurements. To obtain near-dry conditions, 10 mg of 3 Å molecular sieves (8 - 12 mesh beads) per mg of enzyme formulation were added. Samples (30 µl), that were taken in time, were diluted with 450 µl DMSO before HPLC analysis.

After 24 hours the Alcalase formulations were recycled. In the recycling procedure, the Alcalase formulation was first recovered from the reaction liquid. Then, fresh substrates were either added immediately to the Alcalase formulation, or the Alcalase formulation, from which the molecular sieve beads were separated one by one using tweezers, was first rehydrated by washing with successively 1 ml of each Milli-Q, anhydrous *t*-BuOH, and anhydrous THF, before adding fresh substrates and molecular sieve beads.

4.2.6 HPLC analysis

The amounts of dipeptide (Z-Phe-Phe-NH_2), Cam-ester (Z-Phe-OCam), and N-protected phenylalanine (Z-Phe-OH) were analyzed by HPLC (Thermo Separation Products P4000 pump and AS3000 autosampler) using a reversed-phase column (Inertsil ODS-3, C18, 5 µm, 150 × 4.6 mm) at 40 °C. UV detection was done at 220 nm using an Ultimate 3000 Diode Array Detector (Dionex). The gradient program was: 0 - 25 min linear gradient ramp from 5 % to 98 % eluent B, 25 - 29 min linear gradient ramp back to 5 % eluent B, 29 - 40 min 5 % eluent B (eluent A: 0.5 ml l^{-1} methane sulfonic acid (MSA) in Milli-Q, eluent B: 0.5 ml l^{-1} MSA in acetonitrile). The flow was 1 ml min^{-1}. Injection volumes were 20 µl. Quantitative analysis was carried out using calibration curves of Z-Phe-Phe-NH_2, Z-Phe-OCam, and Z-Phe-OH.

4.2.7 Aqueous Alcalase activity

The aqueous activity of the Alcalase formulations was determined by monitoring the hydrolysis of 25 % (v/v) ethyl lactate at 40 °C and pH 6.8 (10 ml of 100 mM sodium phosphate buffer pH 6.8, 20 ml of Milli-Q, and 10 ml of ethyl lactate). The formed lactic acid was titrated with 0.1 mol l^{-1} sodium hydroxide using pH-stat equipment (719 Stat Titrino Metrohm; Herisau, Switzerland). The pH-stat equipment was connected to a computer that logged the consumption of sodium hydroxide every two seconds. The method was based on a protocol obtained from ChiralVision (ChiralVision 2009). The blank consumption of sodium hydroxide was monitored for 10 minutes. Subsequently the Alcalase formulation was added and the consumption of sodium hydroxide was monitored for another 30 minutes. The Alcalase activity is defined by the rate of sodium hydroxide consumption (corrected for the blank consumption of sodium hydroxide). The rate was determined after equilibration and was based on 100 data points in an interval of 200 seconds in total.

4.2.8 Scanning electron microscopy

The Alcalase formulations were placed onto aluminum holders with double-sided sticky carbon tape (EMS, Washington, U.S.A). The samples were sputtered with platinum (JEOL, JFC 1200) and subsequently analyzed with a high resolution scanning electron microscope (FEI, Magellan 400) at room temperature at a working distance of 4.1 - 10.8 mm, with SE detection at 3.5 and 5 kV. Images were optimized by Photoshop CS5.

4.3 Results and discussion

4.3.1 Effect of the initial enzyme hydration state on dipeptide synthesis

The coupling of Z-Phe-OCam and Phe-NH_2 was carried out with eight different Alcalase formulations (Table 4.1). The Alcalase formulations were either applied 'dry' (pre-treated with anhydrous *t*-BuOH and THF), or 'hydrated' (pre-treated with Milli-Q, anhydrous *t*-BuOH, and THF).

The effect of the initial enzyme hydration state on Z-Phe-OCam conversion (Figure 4.2) was studied. Comparing Cam-ester conversion with and without initial

enzyme hydration, it can be concluded that initial enzyme hydration causes a significant increase (*i.e.* no overlap in error bars) in the rates of Z-Phe-OCam conversion in case of Cov, C-OM, C-ST, Dic, S-epo, and S-imi (Figure 4.2). Without initial hydration by Milli-Q washing, these enzymes seem to lack the minimal amount of water needed to maintain their catalytically active conformation (Zaks & Klibanov 1988a; Zaks & Klibanov 1988b; Gaertner & Puigserver 1989; Adlercreutz 1991; Volkin *et al.* 1991; Ljunger *et al.* 1994; Klibanov 2001; Bordusa 2002; Halling 2004; Paravidino *et al.* 2010; Vossenberg *et al.* 2012a). This lack of water and therefore lack of enzyme activity is especially significant for Cov and C-OM. Initial hydration of C-tBu does not cause an increase in the rate of Z-Phe-OCam conversion. Apparently, the crude untreated C-tBu formulation was already sufficiently hydrated.

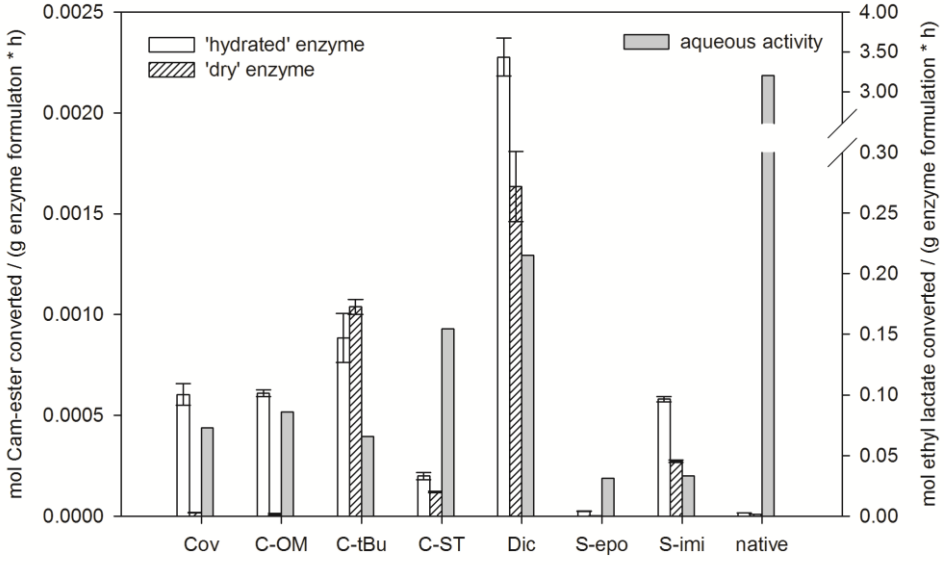

Figure 4.2. Effect of initial hydration state of different Alcalase formulations on the conversion rate of Z-Phe-OCam (left y-axis) and the aqueous activity of the Alcalase formulations (right y-axis). The amount of converted Cam-ester was measured after 1 h of incubation at 25 °C, except for 'dry' Cov, 'dry' C-OM, 'dry' S-epo, and native Alcalase. These formulations were incubated for 24 h. The error bars are equal to the standard deviation of 3 independent measurements.

Only with 'hydrated' C-ST and S-epo, Z-Phe-OCam hydrolysis was observed (in the present system only the hydrolysis of substrate, and not of the product, occurs (Vossenberg *et al.* 2012a)), albeit minimal. Due to the low activity of C-ST and S-epo, a large amount of these enzyme formulations had to be added in order to achieve around 50 % conversion of the Cam-ester in 1 hour. The ratio of molecular sieves to enzyme formulation was therefore the lowest for C-ST and S-epo (respectively 9 and 2.5 mg molecular sieve beads per mg of enzyme formulation). Apparently, the amount of molecular sieves present was not sufficient to adsorb all the water left after washing the enzyme formulations with anhydrous *t*-BuOH and THF, and thus to prevent Cam-ester hydrolysis. For practical reasons (preventing the use of large amounts of molecular sieves) the amount of molecular sieves was not adjusted. With the other enzyme formulations, the Cam-ester was exclusively converted to dipeptide without hydrolysis. In these cases, the amount of molecular sieve beads (> 14 mg per mg of enzyme formulation) was apparently high enough to prevent Z-Phe-OCam hydrolysis.

4.3.2 Comparison of Alcalase formulations

In terms of Cam-ester conversion after 1 h of incubation, Dic, both in 'dry' and 'hydrated' form, is the most active enzyme formulation (Figure 4.2), followed by C-tBu (independent of the hydration state), 'hydrated' Cov, 'hydrated' C-OM, and 'hydrated' S-imi. The least active enzyme formulations for the synthesis of Z-Phe-Phe-NH_2 are native Alcalase, C-ST, and S-epo (Figure 4.2). Although hydration of native Alcalase, by water vapor through the gas phase, increased its synthetic activity, it was probably still too dry for significant enzymatic peptide synthesis in THF. The reason for the low activity of C-ST and S-epo is not clear, especially because the aqueous activity (*i.e.* the hydrolysis of ethyl lactate) of C-ST is 1.7 times higher than the activity of C-OM and the aqueous activities of S-epo and S-imi are similar (Figure 4.2). One might think that the time for the hydration step (10 seconds) may not have been sufficient to hydrate C-ST and S-epo prior to use in organic solvent. We therefore washed C-ST en S-epo for 30 minutes with Milli-Q. The longer hydration step did, however, not remedy the low synthetic activity of C-ST and S-epo in organic solvent at all.

4.3.3 Repeated use of Alcalase formulations

For an Alcalase formulation to be used for dipeptide synthesis in organic media for longer periods of time, the operational stability of the enzyme formulation is important. We therefore studied the repeated use, with and without intermediate rehydration, of the four most active Alcalase formulations, *i.e.* 'hydrated' Cov, C-OM, C-tBu, and Dic (Figure 4.3 A - D). Z-Phe-OCam was converted only to Z-Phe-Phe-NH_2 (thus was not hydrolyzed) for all 4 enzyme formulations. In the current system, data points requiring extended incubation and handling may have been affected somewhat by a certain amount of solvent evaporation (*e.g.* the concentration of Z-Phe-Phe-NH_2 after about 24 hours of incubation is seen to be higher than the initial concentration of Z-Phe-OCam; Figure 4.3 A - D).

Without intermediate rehydration, the rate of Z-Phe-OCam conversion in the second batch was significantly lower than in the first one, for all four Alcalase formulations. This activity loss in time may either be reversible or irreversible. A possible cause for reversible inactivation is dehydration (Fernandes & Halling 2002): enzymes are known to require some essential water to maintain their catalytically active conformation (Zaks & Klibanov 1988a; Zaks & Klibanov 1988b; Gaertner & Puigserver 1989; Adlercreutz 1991; Volkin *et al.* 1991; Ljunger *et al.* 1994; Klibanov 2001; Bordusa 2002; Halling 2004; Paravidino *et al.* 2010; Vossenberg *et al.* 2012a). Because molecular sieves remove water very efficiently, the enzyme formulation may slowly lose essential water and thus activity, as water is transferred from the enzyme to the molecular sieves. Indeed, in previous work, C-OM that had been pre-incubated with molecular sieve powder for 24 h before addition of substrates, showed minimal activity (Vossenberg *et al.* 2012a). If such inactivation by dehydration is reversible, one should be able to restore activity by rehydrating the enzyme formulations before proceeding with the next batch.

Indeed, a nearly full reversal of inactivation was observed for Cov (Figure 4.3 A), C-OM (Figure 4.3 B), and C-tBu (Figure 4.3 C). With intermediate rehydration, comparable activities were achieved for Cov, C-OM, and C-tBu, in the first and second batch. Apparently, the activity loss observed without intermediate rehydration for Cov, C-OM, and C-tBu, is caused by dehydration.

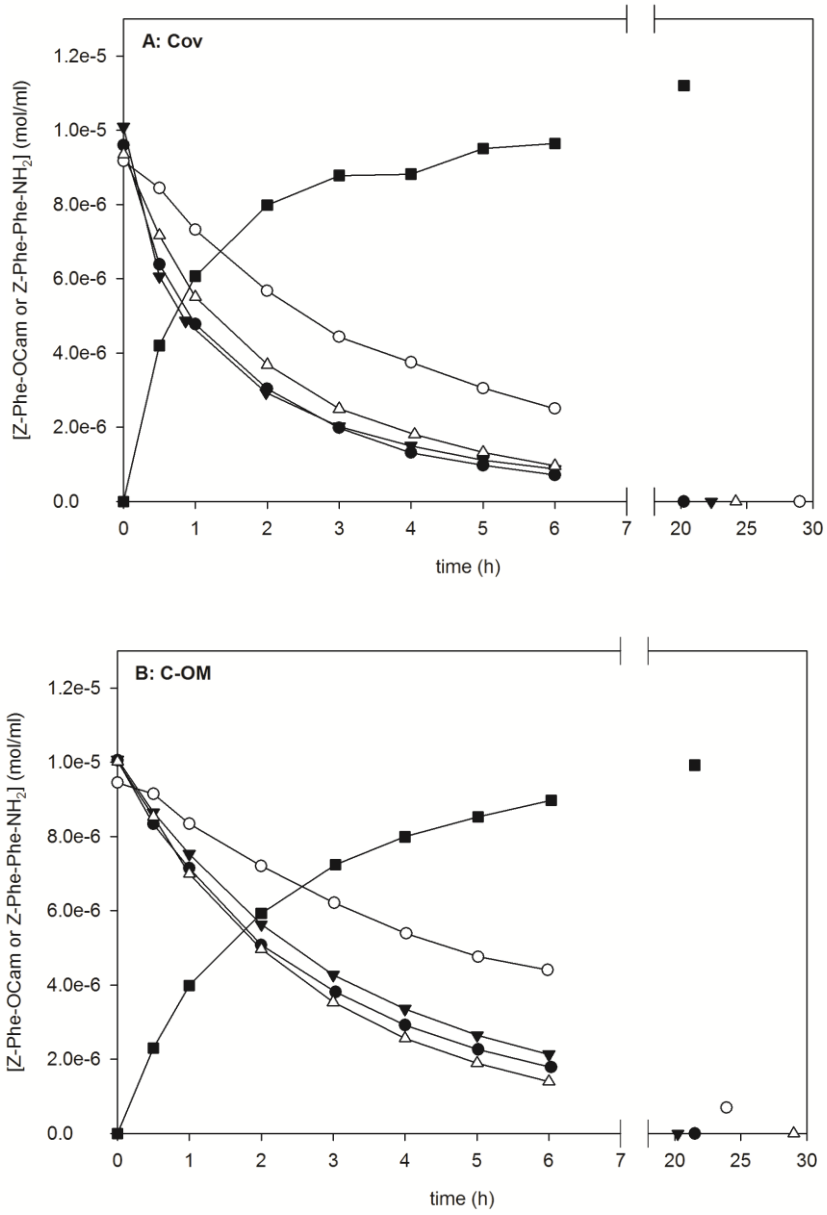

Figure 4.3. Effect of repeated use, with and without intermediate rehydration, of Cov (**A**) and C-OM (**B**) on the conversion of Z-Phe-OCam. Conversion of Z-Phe-OCam (●,▼) and, for illustration, formation of Z-Phe-Phe-NH_2 (■) with an initially 'hydrated' enzyme formulation; subsequent conversion of Z-Phe-OCam, with (Δ) and without (○) intermediate rehydration.

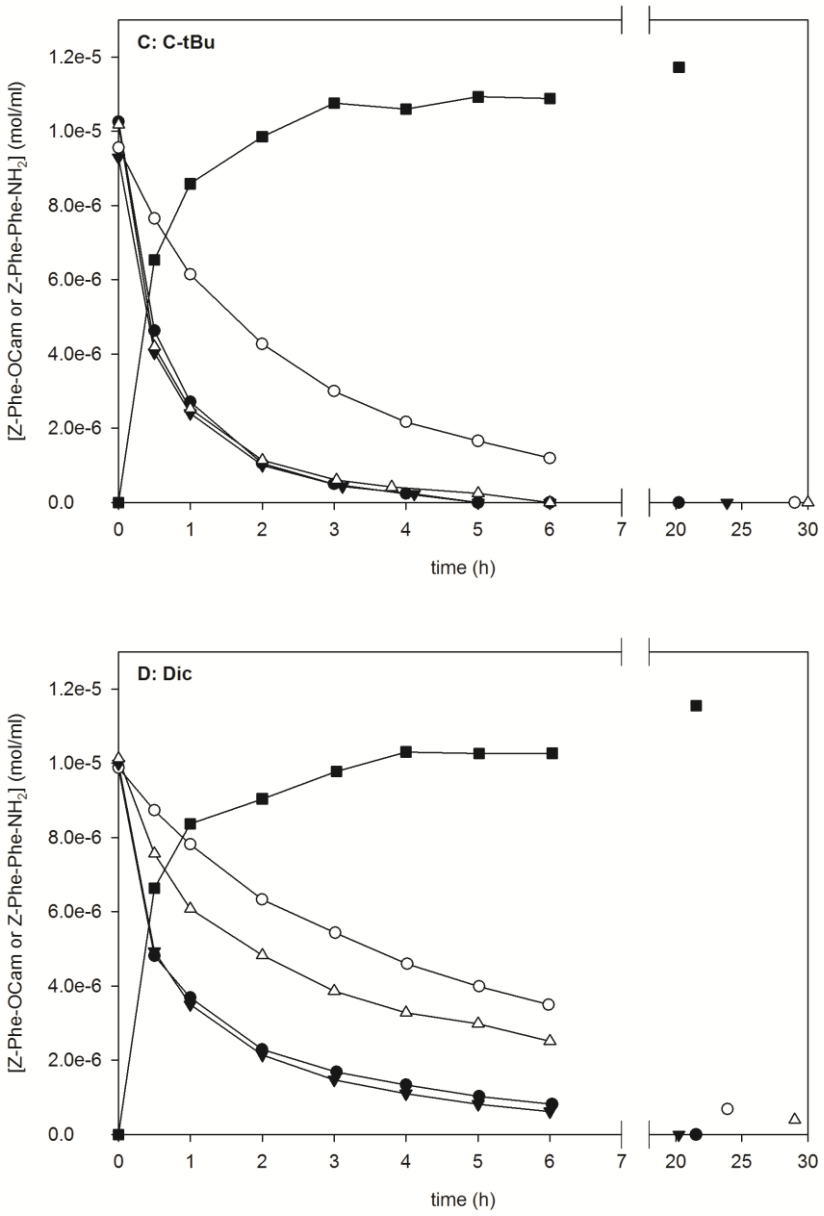

Figure 4.3. Effect of repeated use, with and without intermediate rehydration, of C-tBu (**C**) and Dic (**D**), on the conversion of Z-Phe-OCam. Conversion of Z-Phe-OCam (●,▼) and, for illustration, formation of Z-Phe-Phe-NH_2 (■) with an initially 'hydrated' enzyme formulation; subsequent conversion of Z-Phe-OCam, with (Δ) and without (○) intermediate rehydration.

For Dic (Figure 4.3 D), the inactivation could not be completely reversed by intermediate rehydration. With intermediate rehydration, the activity was higher than without, but still significantly lower than the initial activity in the first batch. Apparently, Dic is partially irreversibly inactivated in THF in the presence of molecular sieves. Also, some Dic may have been lost during the washing steps.

It should be realized that, initially, the 'hydrated' enzyme formulations are clearly not at equilibrium with the dry solvent containing molecular sieves. During the incubation with the substrates and molecular sieves, the system will therefore change and move towards equilibrium, which involves dehydration of the enzyme formulation and thereby loss of enzymatic activity.

The kinetics of de- and re-hydration of the Alcalase formulations as well as the kinetics of the dipeptide synthesis will be studied in more detail in future work.

4.3.4 Evaluation of Alcalase formulations

From the above results it is concluded that initial hydration of Cov, C-OM, C-ST, Dic, S-epo, and S-imi, significantly increases the subsequent rate of Z-Phe-OCam conversion to Z-Phe-Phe-NH_2. In addition, Alcalase immobilized onto dicalite with glutaraldehyde (Dic) is the most active enzyme formulation for dipeptide synthesis (*i.e.* the formulation that initially produces the largest amount of product per gram of total formulation per unit of time).

Although Dic is most active, there are other properties to be considered when selecting an appropriate Alcalase formulation for the synthesis of dipeptides in neat organic media, such as particle size and shape, leaching of enzyme from the support, cost of immobilization method, enzyme loading, and operational stability (Pedersen & Christensen 2000; Guisan 2006). We discuss each of these properties.

An enzyme formulation with a particle size exceeding 100 - 300 μm (Tischer & Kasche 1999; Pedersen & Christensen 2000; Hanefeld *et al.* 2009) can be easily separated from the reaction mixture, which is important when recycling and reusing a costly enzyme formulation and when enzyme contamination of the product should be minimized (Van 't Riet & Tramper 1991; Kragl *et al.* 1999; Tischer & Kasche 1999; Buchholz *et al.* 2005; Sheldon 2007; Hanefeld *et al.* 2009). Apart from native Alcalase, Dic is the finest enzyme formulation (Table 4.1, Figure

4.4). Its small size hampers separation. Separation of Dic from the reaction mixture will therefore be costly (*e.g.* centrifugation). The economic advantage of the good activity of Dic should thus be weighed against its poor separability. Filtration with common sieve plates will work for Cov, S-epo, and S-imi (Table 4.1, Figure 4.4). The economic disadvantage of a lower activity of Cov and S-imi, compared to Dic may thus be compensated by their easier separation.

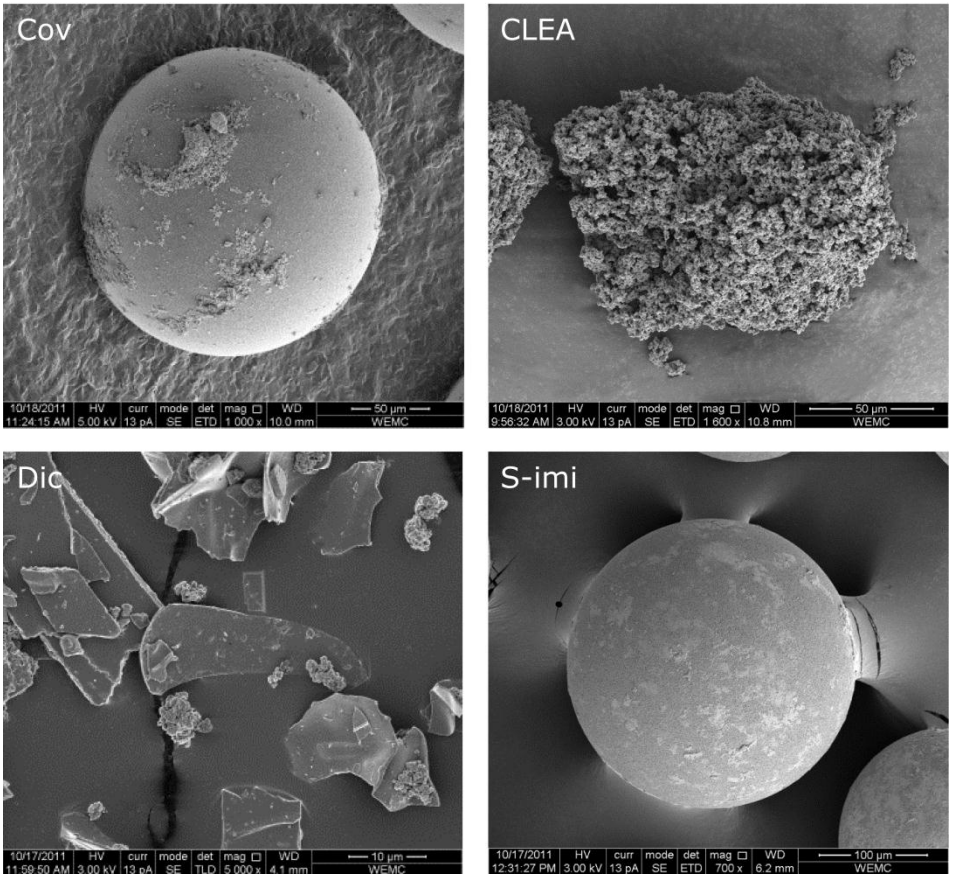

Figure 4.4. Scanning electron microscope images of a Cov bead, a CLEA, Dic particles, and an S-imi bead (an S-epo bead looks identical to an S-imi-bead).

The particle shape of the enzyme formulation is important when the use of a packed-bed reactor is desired. The best candidates for the formation of a bed with good and stable flow properties are rigid and uniform particles that exceed 100 – 200 µm (Buchholz *et al.* 2005). Small, deformable, and irregularly shaped particles can result in channeling, large pressure drops, and clogging (Van 't Riet & Tramper

1991; Xu 2003; Buchholz *et al.* 2005). Cov, S-epo, and S-imi, are the most promising formulations to be used in a packed-bed bioreactor due to their uniformity in shape and size (Figure 4.4).

Enzyme leaching from a support may occur when an enzyme is physically adsorbed onto a carrier (Bickerstaff 1997; Hanefeld *et al.* 2009). Leaching is a minor problem in organic solvents in which the enzymes are insoluble (Pedersen & Christensen 2000; Hanefeld *et al.* 2009). Hydration of the enzyme, however, may aggravate leaching. Dic is prepared by physical adsorbtion of Alcalase onto a carrier and then cross-linking it with glutaraldehyde. Cross-linked enzyme molecules may be trapped inside the pores of the dicalite, and leaching may be modest but not entirely suppressed. Leaching should not occur with Cov, the CLEAs, S-epo, and S-imi, as these enzyme formulations are covalently bound.

The costs of immobilization increase when a carrier is involved (Sheldon 2007). Therefore, the CLEAs are expected to have lower production costs on industrial scale than Cov, Dic, S-epo, and S-imi. On a small scale, the selling price of the CLEAs (€ 225 for 5 grams; information obtained from supplier) is, however, the highest compared to Cov (€ 99 for 5 grams (ChiralVision 2010)), S-epo and S-imi (€ 25 for 5 grams (SPRIN Technologies 2011)).

The costs of an immobilized enzyme formulation increases if a large amount of native enzyme is needed to achieve a certain specific activity of the formulation. The exact enzyme loading can only be quantified during the immobilization procedure, and is unknown for the immobilized Alcalase formulations used in this investigation.

Operational stability of the enzyme formulation in organic solvents is essential when using an enzyme formulation for longer periods of time. Preferably, the activity of the enzyme formulation should not change significantly. If the activity does change, the enzyme formulation needs to be continuously replenished. In this work, the issue of operational stability was investigated for Cov, C-OM, C-tBu, and Dic. When reusing the enzyme formulations, Cov, C-OM, and C-tBu, could reach similar activities in two subsequent batch reactions if subjected to intermediate rehydration. In contrast, Dic is either partially irreversibly inactivated in THF in the presence of molecular sieves or is lost during the washing steps.

To conclude, although Dic is the most active enzyme formulation, it may not be the best choice for dipeptide synthesis in neat organic media on a large scale, due to its small size and lack of operational stability. Cov, C-OM, and C-tBu, may be better choices due to their good operational stability and covalent binding. For application in a packed-bed bioreactor, Cov may be the best choice due to the reasonable size and uniform shape. Therefore, Cov will be the focus of our future work on the kinetics of de- and re-hydration as well as the kinetics of the dipeptide synthesis.

4.4 Conclusions

Initial hydration of Alcalase covalently immobilized onto macroporous acrylic beads (Cov), Alcalase CLEA-OM (C-OM), Alcalase CLEA-ST (C-ST), Alcalase immobilized onto dicalite using glutaraldehyde (Dic), and Alcalase covalently immobilized onto acrylic beads (S-imi and S-epo) causes a significant increase in the rate of Z-Phe-Phe-NH_2 synthesis under dry conditions. Without such initial hydration, the enzymes seem to lack the water needed to maintain their catalytically active conformation.

When reusing Cov, CLEA-OM, CLEA-tBu, and Dic, without intermediate rehydration, a significant decrease in the rate of dipeptide synthesis is observed. The activity loss observed without intermediate rehydration for Cov, C-OM, and C-tBu, was caused by dehydration. Dic is either partially irreversibly inactivated in THF in the presence of molecular sieves or is lost during the washing steps.

Dic is the most active enzyme formulation (the formulation that initially produces the largest amount of product per gram of total formulation per unit of time) for the synthesis of Z-Phe-Phe-NH_2. Nevertheless, it may not be the best choice to synthesize dipeptides in neat organic media on a large scale mainly due to its small size and operational instability.

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CHAPTER 5

Dipeptide synthesis in near-anhydrous organic media: Long-term stability and reusability of immobilized Alcalase

Vossenberg P, Beeftink HH, Nuijens T, Quaedflieg PJLM, Cohen Stuart MA, Tramper J. Dipeptide synthesis in near-anhydrous organic media: Long-term stability and reusability of immobilized Alcalase. Submitted.

Abstract

The long-term stability and reuse of 'hydrated' (water saturated) Alcalase covalently immobilized onto macroporous acrylic beads (Cov) in tetrahydrofuran (THF) were investigated. Cov can be used to synthesize dipeptides under near-anhydrous conditions in THF. Cov was incubated with and without molecular sieves (beads or powder) in THF, in order to investigate whether its stability is affected by the presence of molecular sieves. After different incubation periods in THF, the enzyme activity was determined in an aqueous environment. In addition, Cov was repeatedly recycled in order to examine its reusability. The effect of reuse on the aqueous activity of Cov and on the Cov-catalyzed coupling of phenylalanine amide and the carbamoylmethyl ester of N-protected phenylalanine in near-anhydrous THF was studied. Without molecular sieve beads, Cov hardly inactivated in THF. With molecular sieve beads, Cov lost activity over time. Incubated Cov samples were rotated on a blood rotator, entailing mechanical forces between Cov and the molecular sieve beads. Mechanical damage of Cov by the molecular sieve beads was found to be the main reason for the instability of Cov. During reuse, intermediate rehydration of Cov also caused a small but significant activity loss.

Keywords: *immobilized protease, operational stability, reusability, molecular sieves, near-anhydrous, mechanical damage*

5.1 Introduction

Alcalase (also referred to as subtilisin A and subtilisin Carlsberg) is a protease that may be used in the chemo-enzymatic synthesis of peptides. Peptides play an important role in the fields of health care, nutrition, and cosmetics (Vulfson 1993; Lintner & Peschard 2000; Guzmán *et al.* 2007; Vivó-Sesé & Pla 2007; Stevenson 2009). Alcalase can for instance catalyze the coupling of an amino amide and a chemically synthesized activated N-protected amino acid, *e.g.* an N-protected amino acid carbamoylmethyl (Cam) ester (Miyazawa *et al.* 2002; Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011; Vossenberg *et al.* 2011; Vossenberg *et al.* 2012a; Vossenberg *et al.* 2012b). In previous work we studied the effect of water activity (a_w) on the rate of dipeptide synthesis (Vossenberg *et al.* 2012a). It was found that a carefully chosen amount of molecular sieves was required to prevent hydrolysis, but still allow enzymatic activity; a too large amount of molecular sieves was found to dehydrate and inactivate the enzyme (Vossenberg *et al.* 2012a). Furthermore, we compared different Alcalase formulations with respect to their dipeptide synthesis capability in neat organic solvent in the presence of molecular sieves (*i.e.* under near-anhydrous conditions at very low water activity). Hydration prior to drying (with anhydrous *tert*-butanol (*t*-BuOH) and anhydrous tetrahydrofuran (THF)) of the Alcalase formulations resulted in a significant increase in rate of the subsequent dipeptide synthesis. The most promising enzyme formulation for dipeptide synthesis in organic media on a large scale was found to be hydrated (water saturated) Alcalase covalently immobilized onto macroporous acrylic beads (in this paper abbreviated as Cov) (Vossenberg *et al.* 2012b). This formulation has a reasonable activity with respect to dipeptide synthesis in near-anhydrous organic media, and, from a practical point of view, a reasonable size (150 – 300 μm in diameter (ChiralVision 2011)) and a uniform spherical shape. In addition, Cov features good short-term operational stability in THF as it could be reused at least twice without significant activity loss with respect to dipeptide synthesis (Vossenberg *et al.* 2012b), under the proviso that the enzyme was rehydrated in between subsequent dipeptide synthesis reactions under near-anhydrous conditions.

The present study focuses on the long-term stability and extended reuse of hydrated Cov in THF. The stability of cross-linked subtilisin crystals and native subtilisin Carlsberg in polar solvents has been studied before (Schulze & Klibanov 1991; Fernandes & Halling 2002; Martínez *et al.* 2002). This type of data is, however, not available for the covalently immobilized Alcalase formulation (*i.e.* Cov) we are interested in. Ultimately a process design for peptide synthesis in organic media requires knowledge of and data on the operational stability of the enzyme formulation that was found capable of synthesizing these peptides. Extending the operational stability of an enzyme formulation by minimizing the rate of inactivation requires a mechanistic view on the causes of instability of the enzyme formulation.

The aim of our study was to investigate whether hydrated Cov is stable in dry THF and whether its stability is affected by the presence of molecular sieves. For this, Cov was incubated with and without molecular sieves (beads or powder) in THF and its aqueous activity was subsequently determined. In addition, Cov was repeatedly recycled in order to examine its reusability. The effect of reuse on the aqueous activity of Cov and on the coupling of the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam) and phenylalanine amide (Phe-NH_2) in near-anhydrous THF, catalyzed by Cov, was studied.

5.2 Materials and Methods

5.2.1 Enzymes

Alcalase® covalently immobilized onto macroporous acrylic beads (Cov; ChiralVision, Leiden, The Netherlands) and lyophilized native protease from *Bacillus licheniformis* (also referred to as Alcalase®, subtilisin A, and subtilisin Carlsberg; Sigma-Aldrich, Zwijndrecht, The Netherlands) were used. Cov contains the enzyme Alcalase from Novozymes Corporation (Bagsvaerd, Denmark) (ChiralVision 2010), which is covalently immobilized onto Immobeads 150 (cross-linked copolymer of methacrylate carrying oxirane groups).

5.2.2 Chemicals

All chemicals used were reagent or analytical grade. *t*-BuOH and THF were dried over 3 Å molecular sieves, 8-12 mesh beads (Sigma-Aldrich), for ≥ 1 day prior to use. The molecular sieves were dried at 200 °C and *t*-BuOH was pre-heated to a liquid (40 °C) prior to use. The N-protected Cam-ester of phenylalanine (Z-Phe-OCam) and N-protected phenylalanyl-phenylalanine amide (Z-Phe-Phe-NH_2) were a kind gift from DSM (Geleen, The Netherlands). Phenylalanine amide (Phe-NH_2) and N-protected phenylalanine (Z-Phe-OH) were purchased from Bachem (Weil am Rhein, Germany).

5.2.3 Long-term stability of Cov

Before use, Cov (40 mg) was washed with successively 1 ml of Milli-Q, to initially hydrate the enzyme, and 1 ml of each anhydrous *t*-BuOH and THF, to remove the excess water. A washing step involved adding washing liquid (Milli-Q, *t*-BuOH, or THF) to Cov, shaking the sample for 10 seconds, centrifuging the sample for 2 minutes at 10,000 rpm in order to facilitate the separation of the washing liquid and the enzyme formulation, and removing the washing liquid manually using a pipette. This method was analogous to the procedure used to produce propanol-rinsed enzyme preparations (Partridge *et al.* 1998; Moore *et al.* 2001).

Cov (40 mg) was incubated in 1 ml of anhydrous THF at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm:

1) without molecular sieves,
2) with 10 mg of dry 3 Å molecular sieve beads per mg Cov (in duplo),
3) with 10 mg of dry 3 Å molecular sieve powder (Sigma-Aldrich) per mg Cov (in duplo),
4) with 10 mg pre-hydrated molecular sieve beads per mg Cov. The molecular sieve beads were pre-hydrated by adding them to a vial with Milli-Q for 10 minutes and subsequently removing the excess water using tissue paper.
5) with 17.5 mg pre-hydrated molecular sieve beads per mg Cov.

Cov was also incubated with 10 mg of dry molecular sieve beads per mg Cov without spinning on a blood rotator (in duplo).

After different incubation periods (0 - 32 days), the aqueous activity of the complete content of the Eppendorf tubes (Cov, THF, and molecular sieves) was determined (section '5.2.5 Aqueous Alcalase activity'). For each data point in time a separate Eppendorf tube was incubated, so all time points in the figures represent independent experiments.

5.2.4 Long-term stability of native Alcalase

Native Alcalase (2 mg) was incubated in 1 ml of anhydrous THF at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm. After different incubation periods (0 - 42 days), the aqueous activity of the complete content of the Eppendorf tubes (native Alcalase and THF) was determined (section '5.2.5 Aqueous Alcalase activity'). For each time point a separate Eppendorf tube was incubated.

5.2.5 Aqueous Alcalase activity

The aqueous activity of Alcalase was assayed by monitoring the hydrolysis of 25 % (v/v) ethyl lactate at 40 °C and pH 6.8 (10 ml of 100 mM sodium phosphate buffer pH 6.8, 20 ml of Milli-Q, and 10 ml of ethyl lactate). The resulting lactic acid was titrated with 0.1 mol l^{-1} sodium hydroxide using pH-stat equipment (719 Stat Titrino Metrohm; Herisau, Switzerland). The pH-stat equipment was connected to a computer that logged the consumption of sodium hydroxide every two seconds. The method was based on a protocol obtained from ChiralVision (ChiralVision 2009). The blank consumption of sodium hydroxide was monitored for 10 minutes. Subsequently the Alcalase formulation was added. The Alcalase activity is defined by the rate of sodium hydroxide consumption (corrected for the blank consumption of sodium hydroxide). The rate was based on 100 data points, thus in an interval of 200 seconds in total.

5.2.6 Adsorption of native Alcalase onto molecular sieve beads

Native Alcalase was dissolved in Milli-Q (1 mg ml^{-1}) and 1 ml of the solution was incubated with different amounts of molecular sieve beads (0 - 100 mg) for 24 hours at 25 °C, spinning on a blood rotator at 40 rpm. The same amounts of

beads were also incubated with solely Milli-Q. After 24 hours the vials, containing either native Alcalase and molecular sieve beads, or Milli-Q and molecular sieve beads, were centrifuged for 2 minutes at 10,000 rpm and the UV-absorbance of the supernatant was measured at 280 nm. The UV-absorbance of native Alcalase incubated with molecular sieve beads was corrected for the UV-absorbance of the Milli-Q incubated with molecular sieve beads only.

Native Alcalase (2 mg) was incubated with and without 100 mg molecular sieve beads in 1 ml of anhydrous THF at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm. After 24 hours of incubation, the aqueous activity of native Alcalase was determined (section '5.2.5 Aqueous Alcalase activity').

5.2.7 Calculation of surface areas

The surface area of 100 mg of molecular sieve beads (average diameter: 2 mm; apparent density: 730 kg m^{-3} (Sigma-Aldrich 2011)) available for native Alcalase to adsorb onto, assuming only the outer surface of the 3 Å molecular sieve beads to be available for protein adsorption, is about 4 x 10^{-4} m^2. The 3 Å molecular sieve beads have pore sizes that allow small molecules, such as water, to pass through while larger molecules, such as proteins, cannot (Xinyuan Technology 2012). The surface area required for 1 mg of native Alcalase (density: 1350 kg m^{-3} (Fischer *et al.* 2004)), assuming it to adsorb as a solid monolayer with zero porosity and a thickness equal to the diameter of Alcalase (6.4 nm (Schmitke *et al.* 1997)), to adsorb is about 0.1 m^2.

5.2.8 Settling rate

To get a rough indication of the settling rate of Cov, molecular sieve beads, and molecular sieve powder, they were added separately to 10 ml of THF in a 10 ml test tube of 9 cm in height (Wesselingh *et al.* 1994). After addition of Cov (40 mg), molecular sieve beads (800 mg), or molecular sieve powder (800 mg) to the THF, the mixture was shaken and subsequently allowed to settle. The settling time was measured and from this the settling rate was calculated (Wesselingh *et al.* 1994).

5.2.9 Effect of reuse on the aqueous activity of Cov

Hydrated Cov (40 mg) was incubated in 1 ml of anhydrous THF at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm. Hydrated Cov was obtained by washing with successively 1 ml of each Milli-Q, anhydrous *t*-BuOH, and anhydrous THF. After 24 hours, Cov was rehydrated by washing with successively 1 ml of each Milli-Q, anhydrous *t*-BuOH, and anhydrous THF. After a different number of incubation / washing steps, with a total of 8 recycles, the aqueous activity of Cov was determined (section '5.2.5 Aqueous Alcalase activity'). This was done in duplo. The above procedure was also executed for:

1) Cov incubated in the presence of 10 mg molecular sieve beads per mg Cov. Before rehydration the molecular sieve beads were removed one by one using tweezers. After rehydration, fresh molecular sieve beads were added to Cov.
2) Cov (re)hydrated with 1 ml of 50 mM Tris buffer (pH 8) containing 20 mM calcium chloride instead of Milli-Q, in order to investigate the possible reversible loss of essential calcium ions during washing.
3) Cov washed with Milli-Q, anhydrous *t*-BuOH, and THF, but after the washing step with Milli-Q, Cov was centrifuged for 10 instead of 2 minutes at 10,000 rpm, in order to further facilitate the separation of Milli-Q and Cov.
4) Cov washed with Milli-Q and two times 1 ml of anhydrous THF. The washing step with anhydrous *t*-BuOH was thus omitted.
5) Cov washed with two times 1 ml of anhydrous THF. The washing steps with Milli-Q and anhydrous *t*-BuOH were thus both omitted.

For each number of washes a separate Eppendorf tube was incubated.

5.2.10 Effect of reuse on dipeptide synthesis

Anhydrous THF (900 µl) containing 28.4 µmol Phe-NH_2 and 900 µl anhydrous THF containing 17.5 µmol Z-Phe-OCam were added to 20 mg of hydrated Cov. To hydrate Cov, it was initially washed with 1 ml of Milli-Q and two times 1 ml of anhydrous THF. To obtain very dry conditions, 10 mg molecular sieve beads per mg Cov were added. Samples (30 µl) were taken in time and diluted with 450 µl

dimethyl sulfoxide (DMSO) to stop the reaction before HPLC analysis. The enzymatic reactions were carried out at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm.

After 24 hours, Cov was recycled. The reaction liquid was removed and the molecular sieve beads were removed one by one using tweezers. Cov was then rehydrated by washing with successively 1 ml of Milli-Q and two times 1 ml of anhydrous THF, before adding fresh substrates and molecular sieve beads. Cov was recycled 8 times.

5.2.11 HPLC analysis

The amounts of dipeptide (Z-Phe-Phe-NH_2), Cam-ester (Z-Phe-OCam), and N-protected phenylalanine (Z-Phe-OH) were analyzed by HPLC (Thermo Separation Products P4000 pump and AS3000 autosampler) using a reversed-phase column (Inertsil ODS-3, C18, 5 µm, 150 × 4.6 mm) at 40 °C. UV detection was done at 220 nm using an Ultimate 3000 Diode Array Detector (Dionex). The gradient program was: 0 - 25 min linear gradient ramp from 5 % to 98 % eluent B, 25 -29 min linear gradient ramp back to 5 % eluent B, 29 - 40 min 5 % eluent B (eluent A: 0.5 ml l^{-1} methane sulfonic acid (MSA) in Milli-Q, eluent B: 0.5 ml l^{-1} MSA in acetonitrile). The flow was 1 ml min^{-1}. Injection volumes were 20 µl. Quantitative analysis was carried out using calibration curves of Z-Phe-Phe-NH_2, Z-Phe-OCam, and Z-Phe-OH.

5.3 Results and Discussion

5. 3.1 Long-term stability of Cov

To investigate whether hydrated Cov is stable in dry THF and whether its stability is affected by the presence of water-capturing molecular sieves, Cov was incubated with and without molecular sieves (beads or powder) in anhydrous THF at 25 °C and subsequently its activity was determined in aqueous conditions. This aqueous measurement will compensate for reversible inactivation due to dehydration, if any.

Figure 5.1 shows the remaining aqueous activity (activity compared to initial activity) of Cov incubated with and without molecular sieve beads in THF spinning

on a blood rotator. Without molecular sieve beads, Cov hardly inactivates in THF: after 30 days of incubation the remaining aqueous activity was about 90% (Figure 5.1 A). This is in agreement with the results of Schulze and Klibanov, who reported almost no loss of aqueous activity of native subtilisin Carlsberg when incubated in *tert*-amyl alcohol and in acetonitrile (Schulze & Klibanov 1991).

In contrast to Cov incubated in THF without molecular sieve beads, Cov incubated in the presence of molecular sieve beads, while spinning on a blood rotator, is unstable: after 30 days of incubation, the remaining aqueous activity was about 55 % (Figure 5.1 A).

One can think of different reasons for the inactivation of Cov in the presence of molecular sieve beads in THF. It is possible that (1) Cov is irreversibly dehydrated and thereby inactivated when molecular sieves are present to serve as a high-capacity water sink. It is also possible that (2) enzyme molecules leach from the Cov formulation and adsorb onto the molecular sieves, where they inactivate. Furthermore, (3) the shear, abrasion, and collisions (mechanical forces) between the small and light Immobeads of the Cov formulation (diameter: 150 - 300 µm (ChiralVision 2011)) and the large, heavy, and rough molecular sieve beads (diameter: 1.7 - 2.4 mm (Sigma-Aldrich 2011)), may lead to the inactivation of enzyme molecules on the surface of the Immobeads. This is similar to the way cells are disrupted in a bead mill, in which glass beads are used to create high shearing forces and collisions (Schütte *et al.* 1997).

To investigate whether Cov is irreversibly dehydrated and thereby inactivated by the molecular sieves (Option 1 for inactivation), it was incubated with pre-hydrated molecular sieve beads. Ten mg of pre-hydrated molecular sieve beads per mg Cov caused the same loss of activity as 10 mg of dry molecular sieve beads per mg Cov (Figure 5.1 B). Irreversible dehydration is therefore not a reason for Cov inactivation by molecular sieve beads. The use of molecular sieve powder should then also have led to inactivation by irreversible dehydration. This was not the case as Cov that was incubated with molecular sieve powder was found to be rather stable: 90 % of the initial activity was found to remain after 32 days (Figure 5.1 A). In addition, increasing the amount of pre-hydrated molecular sieve beads per mg Cov to 17.5 mg led to a faster inactivation than using 10 mg of pre-hydrated

molecular sieve beads per mg Cov (Figure 5.1 B). To our knowledge, irreversible dehydration due to the presence of molecular sieves has not been reported elsewhere either. Reversible dehydration due to the presence of molecular sieves, however, has been reported (Monot *et al.* 1991; Wehtje *et al.* 1997; Cerdán *et al.* 1998).

To investigate whether Alcalase leaches from the Cov formulation and then adsorbs onto the molecular sieves (Option 2 for inactivation), native Alcalase (1 mg) was dissolved in Milli-Q (1 ml) and incubated with different amounts of molecular sieve beads for 24 hours at 25 °C, spinning on a blood rotator. The UV-absorbance of native Alcalase incubated with molecular sieve beads did not differ from the UV-absorbance of solely native Alcalase. Native Alcalase thus does not seem to significantly adsorb onto the molecular sieve beads in Milli-Q. To investigate whether Alcalase in THF leaches from the Cov formulation, adsorbs onto the molecular sieves and thereby inactivates, native Alcalase (2 mg) was incubated, with and without molecular sieve beads, in 1 ml of anhydrous THF for 24 hours at 25 °C, spinning on a blood rotator. No difference was found between the aqueous activity of native Alcalase incubated with and without molecular sieve beads. The above mentioned two results were expected when assuming that the molecular sieve beads are not macroporous and that only the outer surface of the 3 Å molecular sieve beads is available for protein adsorption. The outer surface area provided by the molecular sieve beads (100 mg) is sufficient to adsorb only 0.3 % of the native enzyme (section '5.2.7 Calculation of surface areas'). Also the amounts of molecular sieve beads used in combination with Cov provide only a minimal outer surface area for possible leached enzyme molecules from Cov to adsorb onto and thereby inactivate. It is therefore very unlikely that the inactivation of Cov in the presence of molecular sieve beads is due to the adsorption of leached enzyme molecules from Cov onto the molecular sieves. In addition, if enzyme molecules had been adsorbed onto the molecular sieve surface and had thereby inactivated, the use of molecular sieve powder instead of beads, using equivalent amounts (*i.e.* 400 mg), would have led to a higher adsorption of enzyme molecules, due to a larger surface area available for adsorption, and thereby more enzyme would have inactivated. This was not the case.

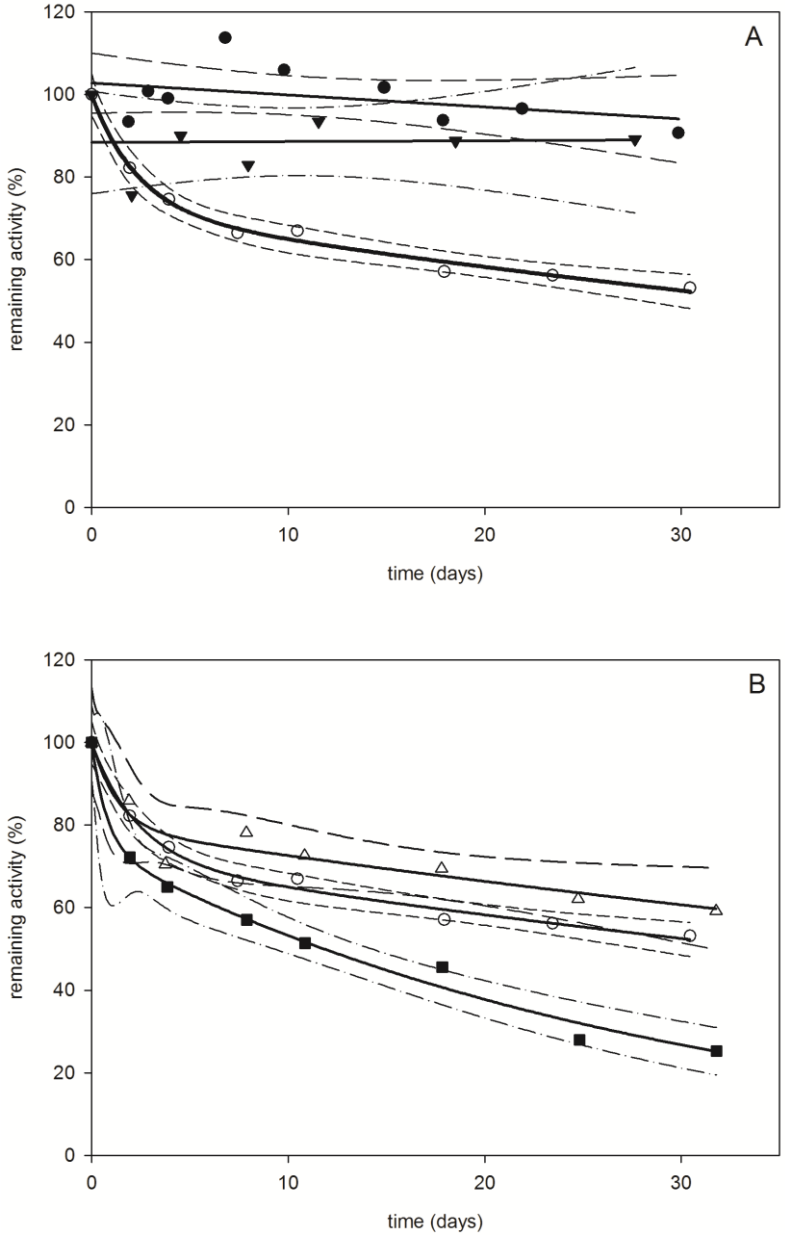


Figure 5.1. Long-term stability of Cov in THF. **A**: Cov without molecular sieve beads (●), with dry molecular sieve beads (10 mg beads per mg Cov) (○), and with dry molecular sieve powder (10 mg powder per mg Cov) (▼). **B**: Cov with dry molecular sieve beads (10 mg beads per mg Cov) (○), with pre-hydrated molecular sieve beads (10 mg beads per mg Cov) (Δ), and with more pre-hydrated molecular sieve beads (17.5 mg beads per mg Cov) (■). 95 % confidence intervals are shown.

The effect of mechanical forces on the activity of Cov (Option 3 for inactivation) was studied by incubating Cov and molecular sieve beads without spinning and therefore without shear and collisions. In addition, Cov was incubated with an equivalent amount of molecular sieve powder, while spinning on a blood rotator. Powder will lead to less shear and less impact per collision than beads due to the small size of the powder particles (3 - 5 μm in diameter (Sigma-Aldrich 2011)). The remaining activity of Cov that was incubated with molecular sieve beads without spinning was around 90 % after 32 days (data not shown). Cov with molecular sieve powder was found to be rather stable (Figure 5.1. A). Apparently, mechanical forces are an important cause for the inactivation of Cov in the presence of molecular sieve beads in this particular system.

The effect of shear on enzyme activity has been reported earlier. Some research groups have observed an effect (Charm & Wong 1970; Tirrell & Middleman 1975; Tirrell & Middleman 1978; Tirrell 1978; Charm & Wong 1981; Ganesh *et al.* 2000; Gunjikar *et al.* 2001; Joshi *et al.* 2001; Bekard *et al.* 2011), whereas others have not (Thomas & Dunnill 1979; Thomas *et al.* 1979; Virkar *et al.* 1980; Harrington *et al.* 1991). The effect of mechanical forces on enzyme activity caused by the presence of molecular sieve beads has, to our knowledge, never been studied in detail. Bovara *et al.* used the combination of molecular sieves and immobilized lipase (adsorbed on Hyflo Super Cel, which is a Celite support), and reused the immobilized lipase several times (by filtering off the enzyme and adding new substrates). They ascribed a reduction in enzyme activity to leakage of enzyme from the support due to mechanical shear by molecular sieves (Bovara *et al.* 1991). In our case, the entire contents of the incubation vials were added to the aqueous activity assay; enzyme loss from the support to the bulk liquid can thus in itself not account for activity losses. Native Alcalase is also not less stable in THF than immobilized Alcalase (data not shown).

In the end, the best way to minimize the inactivation of Cov is to omit the use of molecular sieve beads. Molecular sieves are, however, needed in dipeptide synthesis to prevent hydrolytic side reactions (Vossenberg *et al.* 2012a). Molecular sieve powder instead of molecular sieve beads could be used in dipeptide synthesis because Cov with molecular sieve powder was found to be rather stable.

Molecular sieve beads will be preferred over powder if the molecular sieves need to be separated from Cov. This separation is required in case of recycling of Cov, involving rehydration of Cov and regeneration of the molecular sieves. The molecular sieve beads are significantly larger (diameter: 1.7 - 2.4 mm (Sigma-Aldrich 2011)) than the Cov particles (diameter: 150 - 300 μm (ChiralVision 2011)) and settle very fast (about 150 times faster than Cov; the settling rate is about 9 cm s^{-1} in THF). The molecular sieve powder, however, is very fine (3 - 5 μm in diameter (Sigma-Aldrich 2011)) and settles slowly (the settling rate is about 75 μm s^{-1} in THF, which is about 8 times slower than that of Cov: 600 μm s^{-1} in THF). Nevertheless, in a stirred batch reactor, mechanical damage of Cov by molecular sieve beads will probably be minimal as the particles will not tumble over one another along a wall and they can easily follow the flow of the liquid medium and thus experience little shear.

5.3.2 Effect of reuse on the aqueous activity of Cov

To examine the reusability of Cov, it was repeatedly recycled with intermediate rehydration (consisting of 2 - 3 washing steps). (Re)hydrated Cov was incubated in THF (without substrates), with and without molecular sieve beads at 25 °C while spinning on a blood rotator. After 24 hours, Cov was rehydrated. After each incubation / washing step, with a total of 8 recycles, the aqueous activity of Cov was determined. Cov loses aqueous enzyme activity during each rehydration cycle, with and without molecular sieve beads (Figure 5.2 A). As expected, the rate of Cov activity loss is larger with molecular sieve beads than without due to mechanical forces between Cov and the molecular sieve beads during the incubation time in THF. This activity loss may have been aggravated by the bead manipulation during each rehydration step.

In addition to mechanical forces, there are different potential causes for the loss of enzyme activity. Different options to prevent this loss of enzyme activity were examined. Cov was rehydrated with Tris buffer (pH 8) containing 20 mM $CaCl_2$ instead of Milli-Q (Figure 5.2 A), which did not prevent the activity loss of Cov. The loss of enzyme activity can therefore not be explained by the reversible loss of essential calcium ions (Briedigkeit & Frömmel 1989; Lee & Jang 2001; Srimathi *et*

al. 2006) during washing with Milli-Q. In addition, the loss of activity did not change by prolonging the centrifugation time after washing with Milli-Q, in order to possibly recover small Cov beads (Figure 5.2 A). When replacing the *t*-BuOH washing step with a THF washing step (Figure 5.2 B), however, the rate of activity loss of Cov is significantly reduced; beyond 4 washes there is no overlap in the 95 % confidence intervals of Cov washed with Milli-Q, *t*-BuOH and THF, and of Cov washed with Milli-Q and 2x THF. It thus seems that washing with *t*-BuOH partially inactivates the enzyme, either due to the nature of the solvent or due to the temperature (at 40 °C *t*-BuOH is a liquid) at which the solvent is used. When replacing the washing steps with *t*-BuOH and Milli-Q both by a washing step with THF (Figure 5.2 C), there is no significant further reduction in the activity loss of Cov compared to the activity loss of Cov washed with Milli-Q and 2x THF. It thus seems that some enzyme particles are lost during washing, although the Alcalase in the Cov preparation is said to be covalently immobilized. Nevertheless, washing with Milli-Q and THF cannot easily be circumvented, as a significant activity loss with respect to dipeptide synthesis was observed, when Cov was reused with molecular sieves without intermediate rehydration (Vossenberg *et al.* 2012b).

5.3.3 Effect of reuse on dipeptide synthesis

To investigate the reusability of Cov in dipeptide synthesis, the repetitive batch coupling of Z-Phe-OCam with Phe-NH_2 in THF in the presence of molecular sieve beads, catalyzed by Cov, was studied. The Cam-ester was found to be converted to dipeptide only and was not hydrolyzed under the pertinent conditions investigated.

Based on the results of the long-term stability of Cov in THF in the presence of molecular sieve beads (Figure 5.1) and on the results of the effect of reuse on the aqueous activity of Cov (Figure 5.2), a gradual decrease in the rate of dipeptide synthesis with increasing cycle number was expected. Under the present conditions, however, a decrease in the rate of dipeptide synthesis became significant only after cycle 5 (Figure 5.3). It is rather unexpected that the rate of dipeptide synthesis in cycle 2 is faster than in batch 1. The experiment was therefore repeated, with the result remaining unchanged.

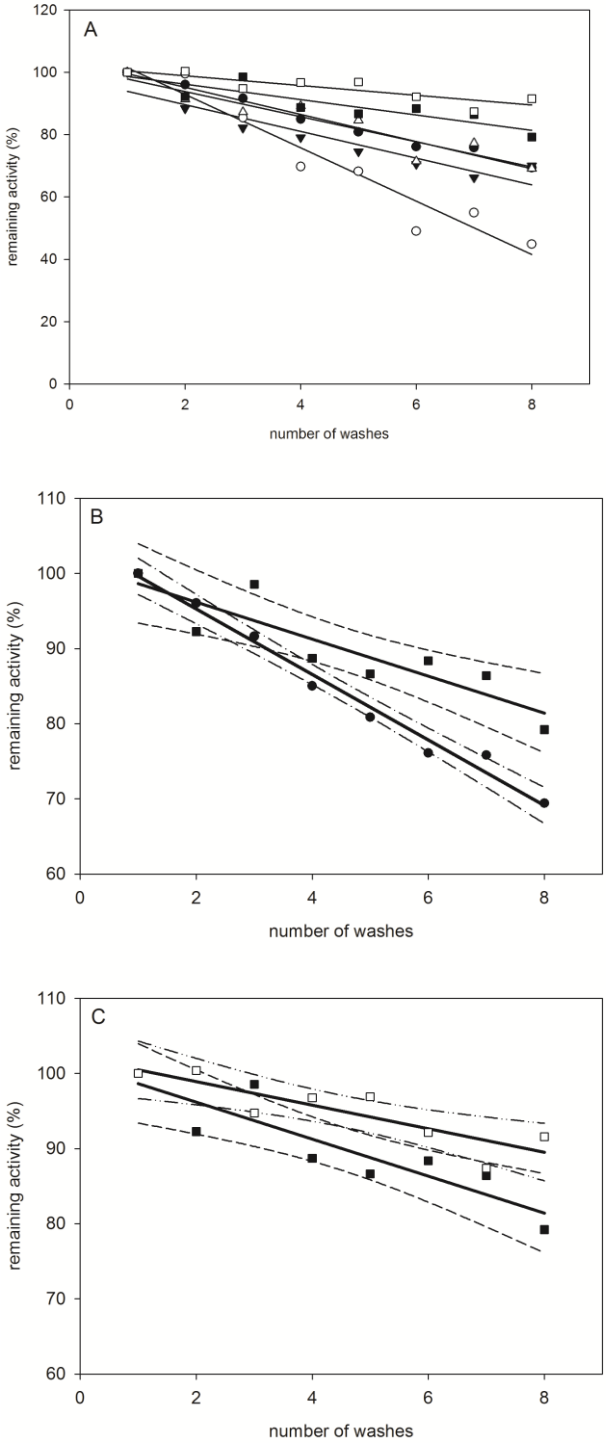


Figure 5.2. Effect of reuse on the aqueous activity of Cov incubated in THF:
- Cov washed with Milli-Q, *t*-BuOH and THF (●);
- Cov incubated with molecular sieve beads, washed with Milli-Q, *t* BuOH and THF (○);
- Cov washed with Tris buffer containing $CaCl_2$, *t*-BuOH and THF (▼);
- Cov washed with Milli-Q, *t*-BuOH and THF, longer centrifugation after Milli-Q wash step (Δ);
- Cov washed with Milli-Q and 2x THF (■);
- Cov washed with 2x THF (□).

A: all data; **B+C**: two series from Figure 5.2 A with 95 % confidence intervals.

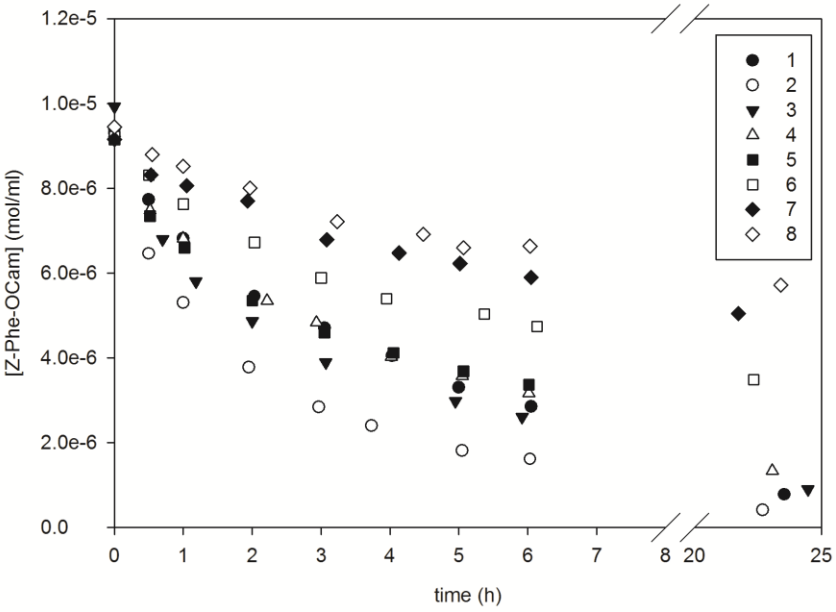


Figure 5.3. Effect of reuse of Cov, with intermediate rehydration, on the conversion of Z-Phe-OCam at 25 °C in time, in the presence of molecular sieve beads.

5.4 Conclusions

Without molecular sieves, Cov hardly inactivated in THF. With molecular sieve beads, while spinning on a blood rotator, Cov lost activity over time. Mechanical forces between Cov and the molecular sieve beads were found to be the main reason for its instability. At the same time, molecular sieves are needed in peptide synthesis to prevent hydrolytic side reactions. In order to reuse Cov for the coupling of Z-Phe-OCam and Phe-NH_2 in the presence of molecular sieves, it needs to be rehydrated in between the batches. Nevertheless, each intermediate rehydration step also caused a small but significant enzyme activity loss.

CHAPTER 6

Kinetics of Alcalase-catalyzed dipeptide synthesis in near-anhydrous organic media

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. Kinetics of Alcalase-catalyzed dipeptide synthesis in near-anhydrous organic media. Submitted.

Abstract

The coupling kinetics of phenylalanine amide and the carbamoylmethyl ester of N-protected phenylalanine in near-anhydrous tetrahydrofuran were investigated. This coupling was catalyzed by Alcalase covalently immobilized onto macroporous acrylic beads; these immobilized enzymes were hydrated prior to use. Near-anhydrous conditions (*i.e.* extremely low water activity) were maintained by a carefully chosen amount of molecular sieve powder. Kinetic characteristics were determined from reaction time courses up to full conversion at various initial concentrations of substrate and product. These progress curve data were fitted with different kinetic models to determine which of these models best approximates the kinetic properties of the immobilized Alcalase with respect to the coupling under study. It was found that the kinetics of the coupling can be described well with a two-substrate kinetic model with two inhibitory products.

Keywords: *enzyme kinetics, progress curve analysis, parameter estimation, product inhibition*

6.1 Introduction

Alcalase can be used in the chemo-enzymatic synthesis of peptides, which are important for health care, nutrition, and cosmetics (Vulfson 1993; Lintner & Peschard 2000; Guzmán *et al.* 2007; Vivó-Sesé & Pla 2007; Stevenson 2009). Alcalase can for instance catalyze the coupling of an amino amide and a chemically synthesized activated N-protected amino acid, *e.g.* an N-protected amino acid carbamoylmethyl (Cam) ester (Miyazawa *et al.* 2002; Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011; Vossenberg *et al.* 2011; Vossenberg *et al.* 2012a; Vossenberg *et al.* 2012b). In the present study the coupling kinetics of phenylalanine amide (Phe-NH_2) and the carbamoylmethyl ester of phenylalanine (of which the amino group was protected using benzyloxycarbonyl, Z-Phe-OCam) in near-anhydrous tetrahydrofuran (THF) were investigated (Figure 6.1). This coupling was catalyzed by Alcalase covalently immobilized onto macroporous acrylic beads (in this paper abbreviated as Cov); these immobilized enzymes were hydrated prior to use. Near-anhydrous conditions were maintained by a carefully chosen amount of molecular sieve powder.

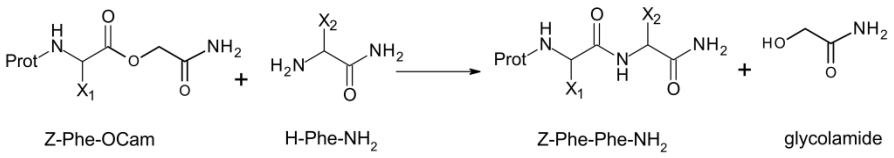


Figure 6.1. Coupling of an N-protected (using benzyloxycarbonyl), C-terminally activated amino acid (the activating group is a carbamoylmethyl) with an amino amide to give a dipeptide and glycolamide. X_1 and X_2 can be any amino acid side chain, but are in this study both a benzyl side chain.

Previously we found that a carefully chosen amount of molecular sieves is required to prevent hydrolysis of the activated substrate (in the present system, only the activated substrate may be hydrolyzed, but not the dipeptide product), as an excess of molecular sieves would dehydrate and inactivate the enzyme (Vossenberg *et al.* 2012a). In addition, we found hydrated Cov to be the most promising enzyme formulation for dipeptide synthesis in organic media on a large scale (Vossenberg *et al.* 2012b). This formulation has a reasonable activity with

respect to dipeptide synthesis in near-anhydrous organic media, and, from a practical point of view, a reasonable size (150 – 300 μm in diameter (ChiralVision 2011)), and a uniform shape. Furthermore, we observed that Cov is stable in THF, with and without molecular sieve powder (Vossenberg *et al.* Submitted-c).

In the present study, we focused on the kinetics of the coupling of Z-Phe-OCam and Phe-NH_2 in THF, catalyzed by Cov. Kinetic characteristics were determined from reaction time courses up to full conversion at various initial concentrations of substrate and product (Reich 1970; Duggleby 1979). These progress curve data were fitted with different kinetic models to determine which model would best approximate the kinetic properties of Cov with respect to the coupling under study. A two-substrate Michaelis-Menten model was used as a starting point, but found inadequate. Several terms were added to obtain a final and satisfactory description of the coupling reaction. Simultaneously, the values of the kinetic parameters associated with the selected model were estimated (Reich 1970; Duggleby 1979).

The kinetics of protease-catalyzed peptide synthesis in monophasic organic solvents has been studied previously, with different proteases such as chymotrypsin (Gaertner & Puigserver 1989; Clapés *et al.* 1990; Clapés *et al.* 1992; Jönsson *et al.* 1995; Sergeeva *et al.* 1997; Vasic-Racki *et al.* 2003) and subtilisin Carlsberg (Sergeeva *et al.* 1997). The present contribution, however, is the first to investigate these kinetics as catalyzed by immobilized Alcalase in THF in the presence of molecular sieves under closely controlled near-anhydrous conditions. In addition, most kinetic studies are based on initial rate measurements, whereas in this study the complete time course of the reaction is modeled. With progress curve analysis, in contrast to initial rate measurements, a complete range of concentrations of the relevant components appear in a single experiment, from which data can be collected (Zavrel *et al.* 2010). A model that is based on this type of experimental data may be expected to predict reactor performance adequately (Bódalo *et al.* 1999). In addition, phenomena occurring after longer time intervals, such as product inhibition, are less likely to be missed with progress curve analysis (Selwyn 1965; Van Boekel 2009; Zavrel *et al.* 2010).

In principle, a single progress curve would suffice to choose a kinetic model and estimate the kinetic parameters, as the concentrations of the relevant components that are consumed or formed by the reaction itself vary inherently (Duggleby 2001). Such a single time course, however, is not very reliable. In the present study, therefore, a range of a starting substrate concentrations was used and combined for analysis.

6.2 Materials and Methods

6.2.1 Enzymes

Alcalase® covalently immobilized onto macroporous acrylic beads (Cov) was purchased from ChiralVision (Leiden, The Netherlands). The formulation contains the enzyme Alcalase from the Novozymes Corporation (Bagsvaerd, Denmark) (ChiralVision 2010). Alcalase is covalently immobilized onto Immobeads 150 (cross-linked copolymer of methacrylate carrying oxirane groups). The Immobeads have a diameter in the range of 150 to 300 µm (ChiralVision 2011).

6.2.2 Chemicals

All chemicals used were reagent or analytical grade. THF was dried over 3 Å molecular sieves, 8 - 12 mesh beads (Sigma-Aldrich, Zwijndrecht, The Netherlands), for at least 1 day prior to use. The molecular sieves were dried at 200 °C, for at least 1 day prior to use. The N-protected Cam-ester of phenylalanine (Z-Phe-OCam) and N-protected phenylalanyl-phenylalanine amide (Z-Phe-Phe-NH_2) were a kind gift from DSM (Geleen, The Netherlands). Phenylalanine amide (Phe-NH_2) and N-protected phenylalanine (Z-Phe-OH) were purchased from Bachem (Weil am Rhein, Germany) and glycolamide ($HOCH_2CONH_2$) from Sigma-Aldrich.

6.2.3 Dipeptide synthesis

Anhydrous THF (900 µl) containing Phe-NH_2 and 900 µl anhydrous THF containing Z-Phe-OCam were added to 20 mg of hydrated Cov. The initial concentration of Phe-NH_2 in the mixture was varied between 1 mM and 34 mM. The initial concentration of Z-Phe-OCam was varied between 5 mM and 11 mM. These initial

substrate concentration combinations resulted in 7 progress curves. Cov was washed with 1 ml of Milli-Q, to initially hydrate the enzyme, and twice with 1 ml of anhydrous THF, to remove the excess water. To obtain dry conditions, 1.5 mg molecular sieve powder per mg of Cov was added. Samples (30 µl) were taken over time for 48 hours and diluted with 450 µl dimethyl sulfoxide (DMSO) to stop the reaction prior to HPLC analysis. Enzymatic reactions were carried out at 25 °C in 2 ml Eppendorf safe-lock tubes on a blood rotator spinning at 40 rpm.

Possible product inhibition was investigated by initially adding a certain amount of dipeptide or glycolamide to a mixture of anhydrous THF containing Phe-NH_2 (16 mM) and Z-Phe-OCam (10 mM). In three experiments, the initial dipeptide concentration was varied between 5 and 8 mM of Z-Phe-Phe-NH_2. In another three experiments, the initial concentration of glycolamide was varied between 7 mM and 18 mM.

All the progress curves in this study were obtained by using the same amount of hydrated Cov (20 mg) and the same amount of molecular sieve powder (1.5 mg molecular sieve powder per mg Cov). Under the conditions studied, this amount of molecular sieve powder is required to prevent hydrolysis of the activated substrate, while still allowing enzymatic activity.

6.2.4 HPLC analysis

Dipeptide (Z-Phe-Phe-NH_2), Cam-ester (Z-Phe-OCam), and N-protected phenylalanine (Z-Phe-OH) were analyzed by HPLC (Thermo Separation Products P4000 pump and AS3000 autosampler) using a reversed-phase column (Inertsil ODS-3, C18, 5 µm, 150 × 4.6 mm) at 40 °C. UV detection was done at 220 nm using an Ultimate 3000 Diode Array Detector (Dionex). The gradient program was: 0 - 5 min linear gradient ramp from 5 % to 98 % eluent B, 25 - 29 min linear gradient ramp back to 5 % eluent B, 29 - 40 min 5 % eluent B (eluent A: 0.5 ml l^{-1} methane sulfonic acid (MSA) in Milli-Q, eluent B: 0.5 ml l^{-1} MSA in acetonitrile). The flow was 1 ml min^{-1}. Injection volumes were 20 µl. Quantitative analysis was carried out using calibration curves of Z-Phe-Phe-NH_2, Z-Phe-OCam, and Z-Phe-OH.

6.2.5 Data correction

In the current system, data points requiring extended incubation and handling were affected somewhat by a certain extent of THF evaporation. All the progress curve data were therefore corrected for this evaporation. In the absence of evaporation, the molar concentrations of Z-Phe-Phe-NH_2 and Z-Phe-OCam at any point in time should equal the initial Z-Phe-OCam concentration. Differences were attributed to evaporation and used to correct for this.

6.2.6 Data analysis

Parameter values in the relevant kinetic rate equation were determined by minimizing the residual sum of squares of the measured concentrations and the concentrations calculated from mass balances, with the Solver function in Microsoft Excel 2010. The mass balances were numerically solved using the Euler method with a time step of 30 seconds, which is minimal compared to the 48 hours of reaction time.

6.3 Results and discussion

All the progress curves involved the same amount of hydrated Cov (20 mg) and the same amount of molecular sieve powder (1.5 mg molecular sieve powder per mg Cov). Under the conditions studied, this amount of molecular sieve powder is required to prevent hydrolysis of the activated substrate, while still allowing enzymatic activity. We previously found that hydrolysis of the dipeptide does not occur in our system (Vossenberg *et al.* 2012a). Furthermore, the conversion of the limiting substrate to dipeptide after about 48 hours ranged from 97 to 100 %, in the progress curves with various initial substrate concentrations. This suggests that Cov is not able to use Z-Phe-Phe-NH_2 and glycolamide as substrates to the produce the protected Z-Phe-OCam and Phe-NH_2. Thus, in our system the reaction (Figure 6.1) proceeds in the forward direction only. Furthermore, Z-Phe-OH is not formed as a byproduct from substrate hydrolysis, unless mentioned otherwise.

The fit procedure was applied to the combined data from several progress curves with various initial substrate concentrations. As the reaction under study is one in which two substrates are converted into two products (Figure 6.1) and in which

both substrates may limit the rate at which product is formed, a two-substrate kinetic model was used to fit the progress curve data,

$$v = v_{\max} \cdot \frac{[A]}{[A] + K_m^A} \cdot \frac{[B]}{[B] + K_m^B} \quad (6.1)$$

where v is the reaction rate (mmol L^{-1} h^{-1}), v_{max} is the maximum reaction rate (mmol L^{-1} h^{-1}), K_m^A is the Michaelis constant (mM) and *[A]* the concentration (mM) of substrate A (here: Z-Phe-OCam), K_m^B is the Michaelis constant (mM) and *[B]* the concentration (mM) of substrate B (here: Phe-NH_2). The reaction is irreversible (Orsi & Tipton 1979). The estimated kinetic parameters associated with the selected model are given in Table 6.2 (column 'substrate Eq. 6.1'), where k_{cat} is the catalytic rate constant (mmol h^{-1} g^{-1}). k_{cat} is calculated by dividing v_{max} by the enzyme concentration (g L^{-1}).

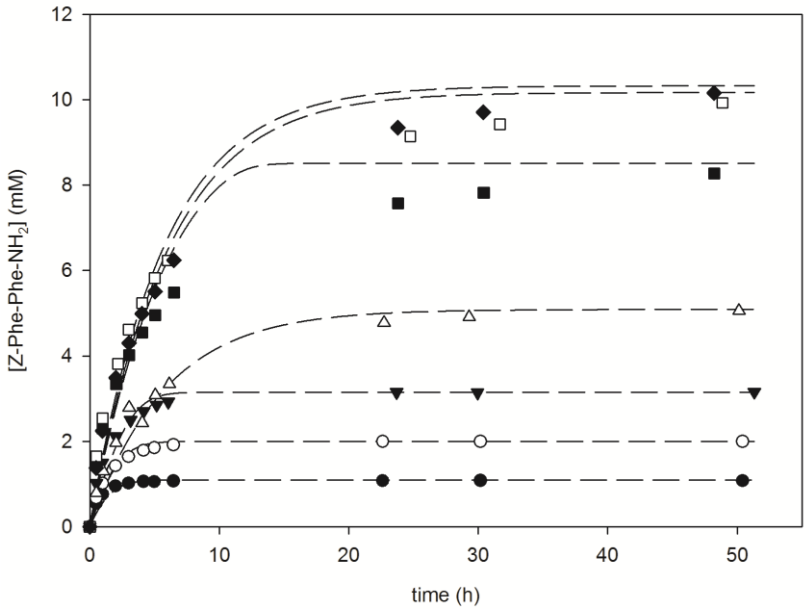


Figure 6.2. Experimental data with various initial substrate concentrations, and fitted progress curves of Z-Phe-Phe-NH_2 synthesis catalyzed by Cov, using a two-substrate kinetic model (Equation 6.1). Legend: Table 6.1. Estimated kinetic parameter values: Table 6.2 (column 'substrate Eq. 6.1').

Table 6.1. Legend of Figures 6.2, 6.3 and 6.6.

Symbol	[Phe-NH_2] (mM)	[Z-Phe-OCam] (mM)
●	1	10
∘	2	10
▼	3	10
Δ	17	5
■	9	11
□	16	10
♦	34	10

The progress curve data with relatively low initial concentrations of the limiting substrate, and therefore low final concentrations of product, could be fitted very well with the two-substrate kinetic model (Figure 6.2). The progress curve data with final product concentrations of more than 5 mM, however, deviated from the model. This deviation was mainly apparent between 5 and 30 hours. In this time period the actual rate of product formation was lower than predicted by the model, which is an indication of product inhibition. In the progress curves with a large excess of Z-Phe-OCam (initial concentration of 10 mM) and a low initial concentration of Phe-NH_2 (1 - 3 mM), some Z-Phe-OH was formed at the end of the experiment, long after the completion of the dipeptide synthesis. The formation of Z-Phe-OH thus did not compete with the formation of Z-Phe-Phe-NH_2.

A two-substrate kinetic model, augmented with competitive product inhibition was used to fit the progress curve data with various initial substrate concentrations (Vasic-Racki *et al.* 2003),

$$v = v_{\max} \cdot \frac{[A]}{[A] + K_m^A \cdot \left(1 + \frac{[P]}{K_i}\right)} \cdot \frac{[B]}{[B] + K_m^B} \qquad (6.2)$$

where K_i is the inhibition constant (mM) and *[P]* the concentration (mM) of the product. The inhibitory product, P, can be either the dipeptide (*i.e.* Z-Phe-Phe-NH_2) or glycolamide, when fitting the progress curve data with various initial substrate concentrations. With variation in the initial substrate concentrations only, a distinction between product inhibition by either the dipeptide or the glycolamide

cannot be made, as both components are formed at equal molar ratios. The estimated kinetic parameters associated with the selected model are given in Table 6.2 (column 'substrate Eq. 6.2'). A two-substrate kinetic model, augmented with non-competitive product inhibition was also tried, but did not fit the progress curve data well.

Table 6.2. Estimated kinetic parameters for Cov-catalyzed dipeptide synthesis in THF.

	Estimated values when fitting data with various initial concentrations of:				
Parameters	substrate Eq. 6.1	substrate Eq. 6.2	dipeptide Eq. 6.2	glycolamide Eq. 6.2	all data Eq. 6.3
K_m^A (mM)	360	600	580	590	2700
K_i (mM)	-	2.2 [1]	4.3 [2]	2.5 [3]	-
K_m^B (mM)	1.4	1.9	1.3	0.75	3.1
v_{max} (mmol L^{-1} h^{-1})	65	240	230	230	1300
k_{cat} (mmol h^{-1} g^{-1})	5.8	22	21	21	110
K_i^P (mM)	-	-	-	-	2.5
K_i^Q (mM)	-	-	-	-	8.9

[1] Inhibitor = dipeptide or glycolamide
[2] Inhibitor = dipeptide
[3] Inhibitor = glycolamide

Evidently, the two-substrate kinetic model considering competitive product inhibition fitted the progress curve data with various initial substrate concentrations better (Figure 6.3) than the former model without product inhibition (Figure 6.2). The reaction rate of the Cov-catalyzed conversion of Z-Phe-OCam and Phe-NH_2 to Z-Phe-Phe-NH_2 is thus affected by product formation. To reduce the effect of the product inhibition on Cov, a reactor could be designed in which the inhibiting product is selectively removed. Nevertheless, before such a reactor can be designed, one needs to determine which of the products, the dipeptide or glycolamide, is the competitive inhibitor. Another option is that both the products inhibit the reaction. A distinction between the dipeptide and glycolamide, with respect to product inhibition, can be made by initially adding various concentrations of either the dipeptide or glycolamide.

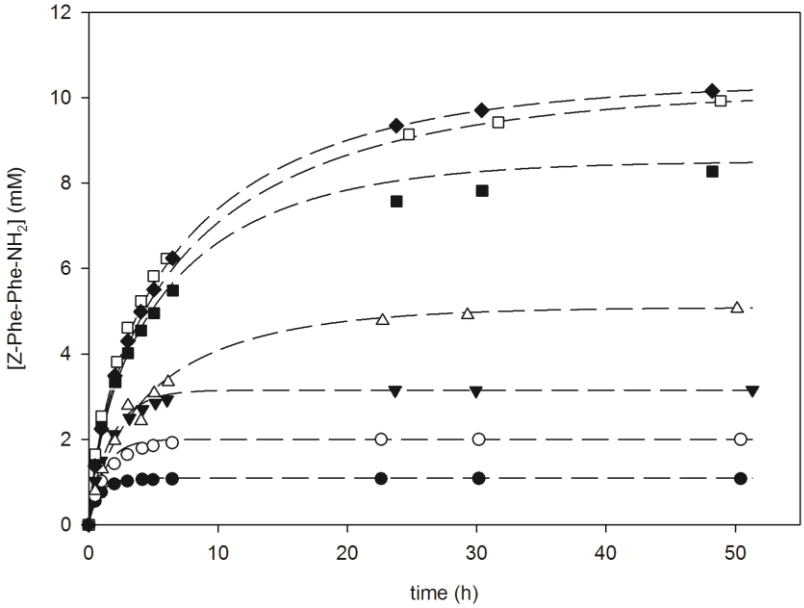


Figure 6.3. Experimental data with various initial substrate concentrations, and fitted progress curves of Z-Phe-Phe-NH_2 synthesis catalyzed by Cov, using a two-substrate kinetic model considering competitive product inhibition (Equation 6.2). Legend: Table 6.1. Estimated kinetic parameter values: Table 6.2 (column 'substrate Eq. 6.2').

The possible product inhibition by the dipeptide was studied by initially adding various concentrations of this substance. The resulting progress curve data were fitted using the same two-substrate kinetic model considering competitive product inhibition as described above (Equation 6.2). In this case *[P]* is the concentration of the dipeptide, assuming that the inhibition is solely caused by the dipeptide. The estimated kinetic parameters associated with the selected model are given in Table 6.2 (column 'dipeptide Eq. 6.2').

During the progress curve experiments, the concentration of Z-Phe-Phe-NH_2 in THF was found to increase up to 16 mM; beyond this concentration, a visible precipitate was observed. The maximum solubility in THF of the other reaction product, glycolamide, was found to lie between 18 and 32 mM. As soon as visible precipitation of Z-Phe-Phe-NH_2 (if any) occurred in the reaction vials, the pertinent experiment was terminated. For some progress curves, therefore, the reaction did not proceed to full completion.

The rate of product formation decreased with increasing amount of initially added dipeptide (Figure 6.4). The Cov-catalyzed dipeptide synthesis was thus inhibited by the dipeptide; not excluding that glycolamide is an additional inhibitor. Due to the finite solubility of the dipeptide in THF, only a limited range of initial dipeptide concentrations could be tested, however. The estimated value for K_i was higher than in the fit of the previous experiments with variable initial substrate concentrations (Table 6.2). Possibly, competitive product inhibition by the dipeptide was initially overestimated, and glycolamide is likely to be an additional inhibitor.

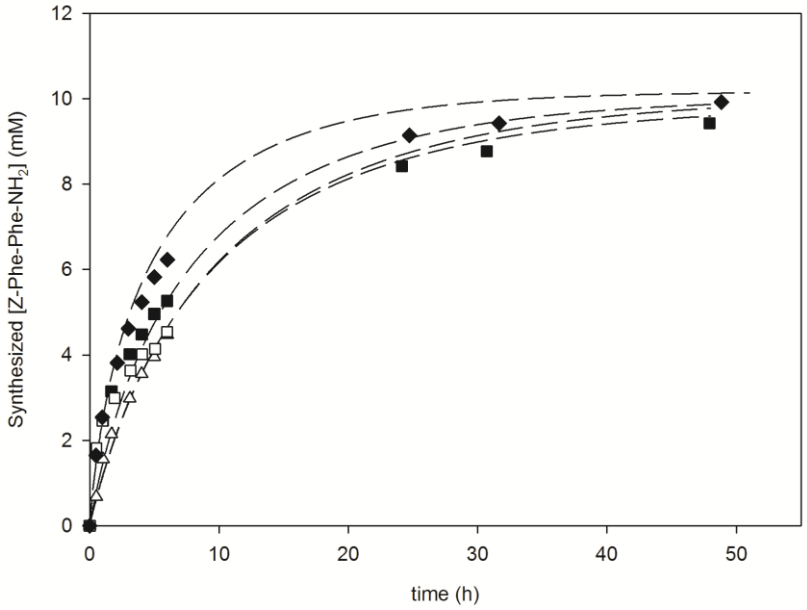


Figure 6.4. Experimental data with various initial dipeptide concentrations (0 ♦, 5 ■, 7 □, and 8 Δ mM dipeptide), and fitted progress curves of Z-Phe-Phe-NH_2 synthesis catalyzed by Cov, using a two-substrate kinetic model considering competitive product inhibition (Equation 6.2), in which the dipeptide is the inhibitor. The dipeptide was initially added to a mixture of anhydrous THF containing Phe-NH_2 (16 mM) and Z-Phe-OCam (10 mM). Estimated kinetic parameter values: Table 6.2 (column 'dipeptide Eq. 6.2').

The possible product inhibition by glycolamide was studied by initially adding various concentrations of this substance. The resulting progress curve data were fitted using the same two-substrate kinetic model considering competitive product inhibition as described above (Equation 6.2). In this case *[P]* is the concentration of

glycolamide, assuming that the inhibition is solely caused by glycolamide. The estimated kinetic parameters associated with the selected model are given in Table 6.2 (column 'glycolamide Eq. 6.2').

The rate of product formation decreased with increasing amount of initially added glycolamide (Figure 6.5). The Cov-catalyzed dipeptide synthesis was thus inhibited by glycolamide.

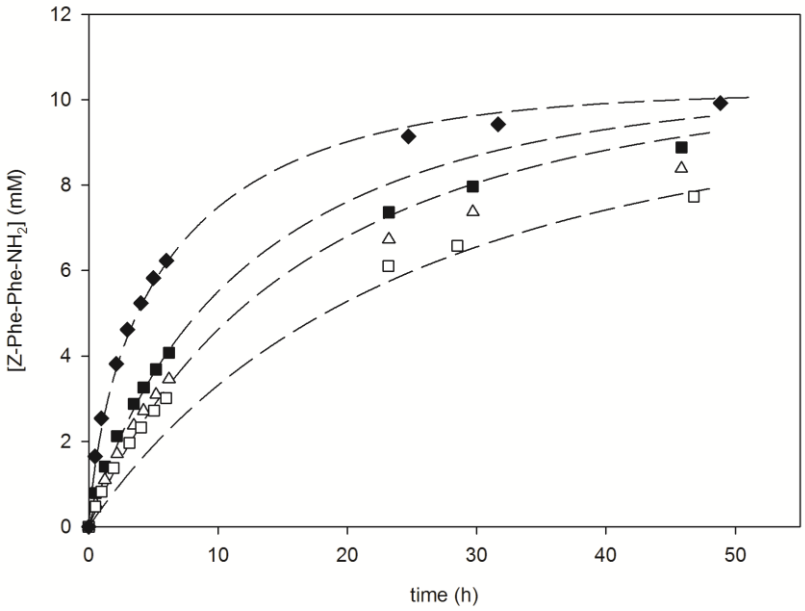


Figure 6.5. Experimental data with various initial glycolamide concentrations (0 ♦, 6 ■, 11 □, and 18 Δ mM glycolamide), and fitted progress curves of Z-Phe-Phe-NH_2 synthesis catalyzed by Cov, using a two-substrate kinetic model considering competitive product inhibition (Equation 6.2), in which glycolamide is the inhibitor. Glycolamide was initially added to a mixture of anhydrous THF containing Phe-NH_2 (16 mM) and Z-Phe-OCam (10 mM). Estimated kinetic parameter values: Table 6.2 (column 'glycolamide Eq. 6.2').

From the experiments in which the concentration of the dipeptide and glycolamide were varied independently, it can be concluded that both the dipeptide and glycolamide are inhibiting the coupling reaction. A two-substrate kinetic model with two competitive product inhibition terms was then used to fit all the progress curve data (*i.e.* the data with various initial substrate, dipeptide and glycolamide concentrations),

$$v = v_{\max} \cdot \frac{[A]}{[A] + K_m^A \cdot \left(1 + \frac{[P]}{K_i^P} + \frac{[Q]}{K_i^Q}\right)} \cdot \frac{[B]}{[B] + K_m^B} \tag{6.3}$$

where *[P]* is the concentration of glycolamide (mM), *[Q]* is the concentration of the dipeptide (mM), and K_i^P and K_i^Q are the respective inhibition constants (mM). The estimated kinetic parameters associated with the selected model are given in Table 6.2 (column 'all data Eq. 6.3').

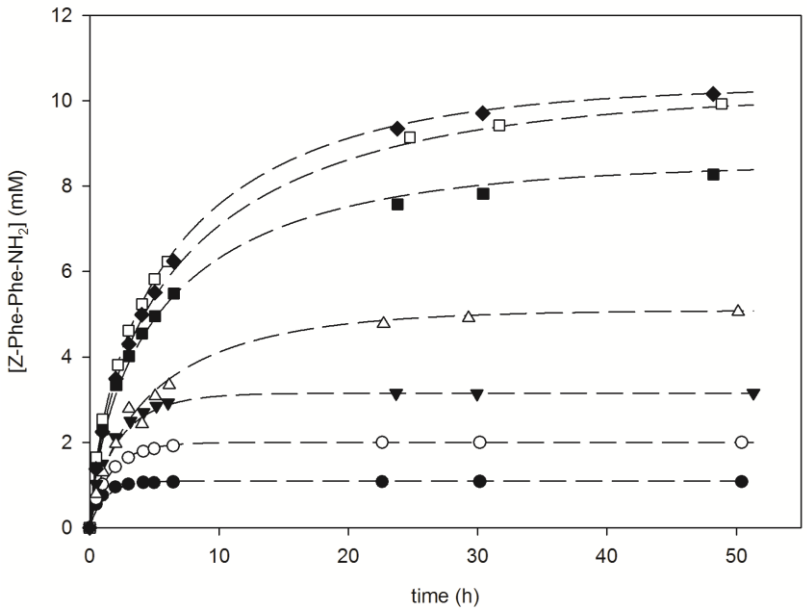


Figure 6.6. Experimental data with various initial substrate concentrations, and fitted progress curves of Z-Phe-Phe-NH_2 synthesis catalyzed by Cov, using a two-substrate kinetic model with two competitive product inhibition terms (Equation 6.3). Legend: Table 6.1. Estimated kinetic parameter values: Table 6.2 (column 'all data Eq. 6.3').

All the progress curve data can be fitted best (*i.e.* gives the lowest sum of squares of the difference between the measured value of the dipeptide and that expected from the equation) with the two-substrate kinetic model with two competitive product inhibition terms. Figure 6.6 shows this best fit and the experimental data with various initial substrate concentrations. Glycolamide (P) inhibits the dipeptide synthesis more strongly than the dipeptide (Q), as $K_i^P < K_i^Q$ (Table 6.2, column 'all data Eq. 6.3'). Nevertheless, both compounds are significant inhibitors

when synthesizing dipeptides near the solubility limit of the dipeptide in THF (ca. 16 mM), which will likely be the practical concentration range at which dipeptides will be synthesized.

The synthesis of Z-Phe-Phe-NH_2, starting with 15 mM of both substrates, was simulated for four different scenarios: (1) glycolamide and the dipeptide accumulate, (2) the dipeptide is selectively and continuously removed, (3) glycolamide is selectively and continuously removed, and (4) both the dipeptide and glycolamide are selectively and continuously removed during synthesis (Figure 6.7). The two-substrate kinetic model with two competitive product inhibition terms was used for this simulation, together with the kinetic parameters that are given in Table 6.2 (column 'all data Eq. 6.3'). It can be clearly seen that to reduce the effect of product inhibition on Cov, a reactor should be designed in which at least glycolamide is selectively removed.

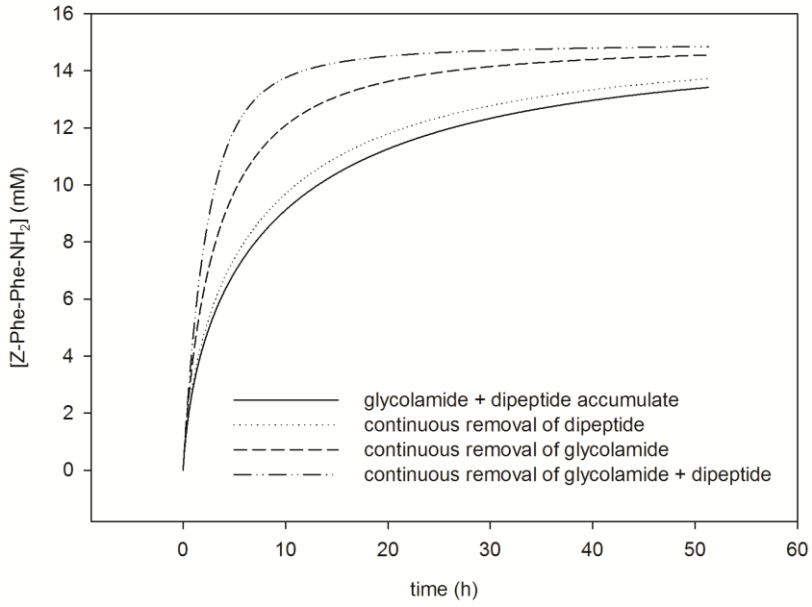


Figure 6.7. Simulation of Z-Phe-Phe-NH_2 synthesis for different scenarios, using a two-substrate kinetic model with two competitive product inhibition terms (Equation 6.3). Used kinetic parameter values: Table 6.2 (column 'all data Eq. 6.3').

In all the kinetic models used in this study to fit the progress curve data, dehydration-induced inactivation of Cov by the molecular sieve powder was

ignored. Yet, as we have shown previously, the repeated use of Cov, in the presence of molecular sieves, without intermediate rehydration leads to the dehydration and thereby inactivation of Cov (Vossenberg *et al.* 2012b). One would think that dehydration of Cov also takes place within a single run up to full conversion of the substrate.

Judging from the quality of fit of the progress curve data with the two-substrate kinetic model with two competitive product inhibition terms, we conclude it is not likely that the dehydration of Cov during the reaction time course in the presence of 1.5 mg molecular sieve powder per mg Cov is significant. However, it is possible that the dehydration is hidden in the estimated kinetic parameter values. The dehydration kinetics of Cov in the presence of molecular sieve powder is presently being studied in more detail and will be submitted in due course.

6.4 Conclusions

The kinetics of the coupling of Z-Phe-OCam and Phe-NH_2 in THF catalyzed by Cov in the presence of molecular sieves were found to obey a two-substrate kinetic model with two competitive product inhibition terms. To reduce the effect of the product inhibition on Cov, a reactor should be designed in which at least glycolamide is selectively removed, as it was found to be the strongest inhibitor.

CHAPTER 7

Effect of enzyme dehydration on Alcalase-catalyzed dipeptide synthesis in near-anhydrous organic media

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. Effect of enzyme dehydration on Alcalase-catalyzed dipeptide synthesis in near-anhydrous organic media. Submitted.

Abstract

The effect of enzyme dehydration by molecular sieves on the coupling of phenylalanine amide and the carbamoylmethyl ester of N-protected phenylalanine in near-anhydrous tetrahydrofuran was investigated. This coupling was catalyzed by Alcalase covalently immobilized onto macroporous acrylic beads (Cov); these immobilized enzymes were hydrated prior to use. The dehydration kinetics of Cov by molecular sieve powder were determined by incubating Cov with different amounts of molecular sieve powder for different periods of time (0 – 80 h). Subsequently, the remaining coupling activity of Cov was measured. Dehydration-induced inactivation of Cov by molecular sieve powder was found to occur in three phases: (1) an initial, rapid, major dehydration-induced inactivation that takes place during the first activity measurement (1 h), (2) a phase of first-order inactivation (20 h), and (3) a relatively low plateau phase in activity. These dehydration kinetics were incorporated into a previously established reaction kinetics model. The resulting model was then used to fit progress curve data of the coupling in the presence of different amounts of molecular sieve powder. Using the estimated parameter values, the model was used to predict independent data sets and found to work well.

Keywords: *enzyme dehydration, enzyme kinetics, progress curve analysis, parameter estimation, dipeptide synthesis, near-anhydrous*

7.1 Introduction

Alcalase can be used in the chemo-enzymatic synthesis of peptides, which are important for health care, nutrition, and cosmetics (Vulfson 1993; Lintner & Peschard 2000; Guzmán *et al.* 2007; Vivó-Sesé & Pla 2007; Stevenson 2009). Alcalase can catalyze the coupling of an amino amide and a chemically synthesized activated N-protected amino acid, such as an N-protected amino acid carbamoylmethyl (Cam) ester (Miyazawa *et al.* 2002; Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011; Vossenberg *et al.* 2011; Vossenberg *et al.* 2012a; Vossenberg *et al.* 2012b). In the present study the effect of enzyme dehydration by molecular sieves on the coupling of phenylalanine amide (Phe-NH_2) and the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam) in near-anhydrous tetrahydrofuran (THF) was investigated. This coupling was catalyzed by Alcalase covalently immobilized onto macroporous acrylic beads (in this paper abbreviated as Cov), these immobilized enzymes were hydrated prior to use. Near-anhydrous conditions were maintained by different amounts of molecular sieve powder.

Previously we found that molecular sieves are required to prevent hydrolysis of the activated substrate (in the present system, only the activated substrate may be hydrolyzed, but not the dipeptide product) (Vossenberg *et al.* 2012a). Nevertheless, an excess amount of molecular sieves or the use of molecular sieves over longer periods of time, dehydrate the enzyme and thereby inactivate it (Vossenberg *et al.* 2012a; Vossenberg *et al.* 2012b). This dehydration-induced inactivation can, however, be counteracted by intermediate rehydration (Vossenberg *et al.* 2012b). Dehydration-induced inactivation is thus a reversible process. Molecular sieves have been used in biocatalytic reactions by other research groups as well (Ergan *et al.* 1988; Ergan *et al.* 1990; Monot *et al.* 1991; Lima *et al.* 1995; Cerdán *et al.* 1998; Nuijens *et al.* 2009; Quaedflieg *et al.* 2010). Nevertheless, the dehydration kinetics of an enzyme due to the presence of molecular sieves was to our knowledge not studied in detail before.

Previously we also found hydrated Cov to be the most promising enzyme formulation for dipeptide synthesis in organic media on a large scale (Vossenberg

et al. 2012b). This formulation has a reasonable activity with respect to dipeptide synthesis in near-anhydrous organic media, and, from a practical point of view, a reasonable size (150 – 300 μm in diameter (ChiralVision 2011)), and a uniform spherical shape. Furthermore, we observed that Cov is stable in THF, with and without molecular sieve powder (Vossenberg *et al.* Submitted-c).

In the present study, we focused on the effect of enzyme dehydration on the rate of coupling of Z-Phe-OCam and Phe-NH_2 in THF, catalyzed by Cov. The dehydration kinetics of Cov in the presence of different amounts of molecular sieve powder were determined by incubating Cov with molecular sieve powder for different periods of time (0 – 80 h), and subsequently measuring the remaining activity of Cov. The resulting dehydration kinetics were incorporated into the previously established reaction kinetics model (Vossenberg *et al.* Submitted-b). It was found that the kinetics of the coupling can be described well with a two-substrate kinetic model with two inhibitory products.

The new model, which simultaneously incorporates the coupling kinetics and the dehydration kinetics, was fitted to progress curve data of the coupling in the presence of different amounts of molecular sieve powder. The resulting model and its parameter values were validated over longer periods of time by recycling Cov multiple times with a certain amount of molecular sieve powder.

7.2 Materials and Methods

7.2.1 Enzymes

Alcalase® covalently immobilized onto macroporous acrylic beads (Cov) was purchased from ChiralVision (Leiden, The Netherlands). The formulation contains the enzyme Alcalase from the Novozymes Corporation (Bagsvaerd, Denmark) (ChiralVision 2010). Alcalase is covalently immobilized onto Immobeads 150 (cross-linked copolymer of methacrylate carrying oxirane groups). The Immobeads have a diameter in the range of 150 to 300 μm (ChiralVision 2011).

7.2.2 Chemicals

All chemicals used were reagent or analytical grade. THF was dried over 3 Å molecular sieves, 8-12 mesh beads (Sigma-Aldrich, Zwijndrecht, The Netherlands),

for at least 1 day prior to use. Molecular sieves were dried at 200 °C, for at least 1 day prior to use. The N-protected Cam-ester of phenylalanine (Z-Phe-OCam) and N-protected phenylalanyl-phenylalanine amide (Z-Phe-Phe-NH_2) were a kind gift from DSM (Geleen, The Netherlands). Phenylalanine amide (Phe-NH_2) and N-protected phenylalanine (Z-Phe-OH) were purchased from Bachem (Weil am Rhein, Germany).

7.2.3 *Dehydration kinetics*

The dehydration kinetics of Cov in the presence of different amounts of molecular sieve powder were determined by incubating Cov with molecular sieve powder for different periods of time and subsequently measuring the remaining activity of Cov with respect to the coupling under study.

Cov was washed with 1 ml of Milli-Q, to initially hydrate the enzyme, and twice with 1 ml of anhydrous THF, to remove the excess water. The hydrated Cov (11 mg) was incubated for different periods of time (0 – 80 h) with different amounts of molecular sieve powder (1.5, 3, 4, 6, and 12 mg molecular sieve powder per mg Cov) and 150 µl of anhydrous THF, at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm. After the incubation time (0 – 80 h), the remaining activity of Cov, with respect to the coupling under study, was measured by adding anhydrous THF (150 µl), containing 4.8 µmol Phe-NH_2 and 2.9 µmol Z-Phe-OCam, to the reaction vial. After 1 h of reaction time, a sample (50 µl) was taken and diluted with 700 µl dimethyl sulfoxide (DMSO) to stop the reaction prior to HPLC analysis of the reaction ingredients.

7.2.4 Dipeptide synthesis

Anhydrous THF (900 µl) containing Phe-NH_2 and 900 µl anhydrous THF containing Z-Phe-OCam were added to 20 mg of hydrated Cov. The initial concentration of Phe-NH_2 was 16 mM. The initial concentration of Z-Phe-OCam was either 5 mM or 10 mM. Different amounts of molecular sieve powder (1.5, 3, 4, 6, and 12 mg molecular sieve powder per mg Cov) were added to the reaction vial. Samples (30 µl) were taken over time for 48 hours and diluted with 450 µl DMSO to stop the reaction prior to the HPLC analysis that was used to assess the reaction

progress. Enzymatic reactions were carried out at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm.

7.2.5 Repeated dipeptide synthesis

Anhydrous THF (900 µl) containing Phe-NH_2 and 900 µl anhydrous THF containing Z-Phe-OCam were added to 20 mg of hydrated Cov. The initial concentration of Phe-NH_2 was 15 mM and of Z-Phe-OCam 9 mM. Molecular sieve powder (6 mg per mg Cov) was added to the reaction vial. Samples (30 µl) were taken over time for 24 hours and diluted with 450 µl DMSO to stop the reaction prior to HPLC analysis. Enzymatic reactions were carried out at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm. After 24 hours, Cov was recycled. The reaction vial was centrifuged for 2 minutes at 10,000 rpm, after which the reaction liquid was removed from Cov and the molecular sieve powder. Fresh substrates were added. Cov was reused for 5 times.

7.2.6 HPLC analysis

Dipeptide (Z-Phe-Phe-NH_2), Cam-ester (Z-Phe-OCam), and N-protected phenylalanine (Z-Phe-OH) were analyzed by HPLC (Thermo Separation Products P4000 pump and AS3000 autosampler) using a reversed-phase column (Inertsil ODS-3, C18, 5 µm, 150 × 4.6 mm) at 40 °C. UV detection was done at 220 nm using an Ultimate 3000 Diode Array Detector (Dionex). The gradient program was: 0 - 5 min linear gradient ramp from 5% to 98% eluent B, 25 - 29 min linear gradient ramp back to 5% eluent B, 29 - 40 min 5% eluent B (eluent A: 0.5 ml l^{-1} methane sulfonic acid (MSA) in Milli-Q, eluent B: 0.5 ml l^{-1} MSA in acetonitrile). The flow was 1 ml min^{-1}. Injection volumes were 20 µl. Quantitative analysis was carried out using calibration curves of Z-Phe-Phe-NH_2, Z-Phe-OCam, and Z-Phe-OH.

7.2.7 Data correction

In the current system, data points requiring extended incubation and handling were affected somewhat by a certain extent of THF evaporation. All the progress curve data were therefore corrected for this evaporation. In the absence of evaporation, the molar concentrations of Z-Phe-Phe-NH_2 and Z-Phe-OCam at any

point in time should equal the initial Z-Phe-OCam concentration. Differences were attributed to evaporation and used to correct for this.

7.2.8 Data analysis

Parameter values in the relevant kinetic rate equation were determined by minimizing the residual sum of squares of the measured concentrations and the concentrations calculated from mass balances, with the Solver function in Microsoft Excel 2010. The mass balances were numerically solved using the Euler method with a time step of 30 seconds, which is minimal compared to the 24 hours of reaction time.

7.3 Results and Discussion

The kinetics of dehydration of Cov in the presence of five different amounts of molecular sieve powder were determined by incubating Cov with molecular sieve powder for different periods of time (0 – 80 h), and subsequently measuring the remaining coupling activity of Cov. The residual activity was determined by adding both amino acids to the incubated Cov and measuring the amount of synthesized dipeptide after 1 h. During this activity measurement the molecular sieves remain present. Figure 7.1 shows the residual coupling activity as a function of the incubation time with molecular sieve powder. For clarity reasons, the results for only three amounts of molecular sieve powder (1.5, 4, and 12 mg powder per mg Cov) are shown. In principle, the activated substrate may be hydrolyzed; such hydrolysis, however, was not observed in any of the present dehydration measurements. As found previously (Vossenberg *et al.* 2012a), also the current dipeptide product is not hydrolyzed.

The initial amount of synthesized dipeptide (*i.e.* after 1 h of reaction time, without any incubation time with molecular sieves before the activity measurement) was significantly lower with a larger amount of molecular sieve powder added (Figure 7.1). A major dehydration-induced inactivation of Cov by the molecular sieve powder thus already takes place during the 1 h of activity measurement, *i.e.* immediately upon addition of the molecular sieve powder.

With an increase in incubation time with molecular sieves, Cov seemed to inactivate in two phases: one phase lasting up to 20 hours in which part of the total amount of Cov inactivates relatively fast and a second phase in which a virtually persistent part of Cov inactivates only minimally, if at all.

The persistent level of residual activity of Cov is observed to be lower with a larger amount of molecular sieve powder (Figure 7.1). We believe that at this point an equilibrium in water is reached between the molecular sieves and Cov itself. With a larger amount of molecular sieve powder, more water will bound by the molecular sieve powder and less water will be available for Cov, leading to a decrease in activity.

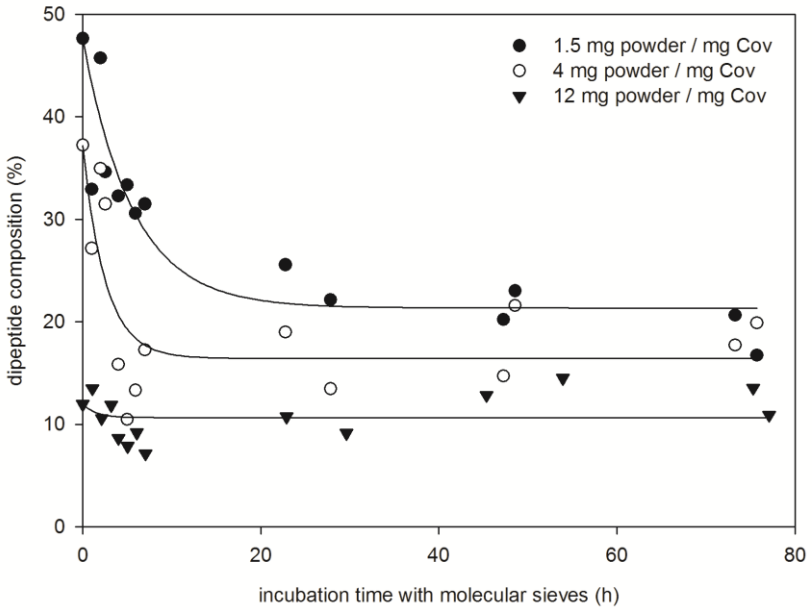


Figure 7.1. Dipeptide composition after 1 h of reaction time versus the incubation time with molecular sieve powder. Data were fitted with Equation 7.2.

The data were fitted with an empirical double exponential decay function with 3 parameters, *i.e.* x_1, k_1, and k_2,

$$\frac{A_t}{A_0} = x_1 e^{-k_1 t} + (1 - x_1) e^{-k_2 t} \tag{7.1}$$

This type of exponential decay function has been used to describe the thermal inactivation of enzymes (Henley & Sadana 1986; Ulbrich *et al.* 1986; Arroyo *et al.* 1999; Aymard & Belarbi 2000). The double exponential decay function is actually a simplification from a more general enzyme decay function that assumes a continuous distribution of inactivation rates. The occurrence of several enzyme populations is not unlikely for immobilized enzymes because there is a heterogeneity in enzyme orientation, location of the enzyme in the carrier, and number of linkages between the enzyme and the carrier (Ulbrich *et al.* 1986). In this case, it can be imagined that there are enzyme populations in the Cov formulation that differ in their susceptibility to dehydration-induced inactivation. x_1 may be the fraction of the enzyme population that inactivates with a rate constant k_1 (h^{-1}), and 1- x_1 the fraction of the enzyme population that inactivates with a rate constant k_2 (h^{-1}). A_t is the enzyme activity at time t (h) and A_0 the starting enzyme activity after the initial dehydration-induced inactivation (due to the dehydration-induced inactivation during the first activity measurement (Figure 7.1)). A_0 is dependent on the amount of molecular sieve powder: A_0 decreases with an increasing amount of molecular sieve powder.

After preliminary fitting of the data set with Equation 7.1, the resulting k_2 value virtually equaled zero. Consequently, the double exponential decay function was reduced to a single exponential decay function with 2 parameters, *i.e.* x_1 and k_1,

$$\frac{A_t}{A_0} = x_1 e^{-k_1 t} + (1 - x_1) \qquad (7.2)$$

All the dehydration data were fit with Equation 7.2. The resulting parameters, x_1 and k_1, are listed in Table 7.1. It should be noted, however, that the persistent levels of residual activity of Cov in Figure 7.1 are not equal to 1-x_1, for this A_0 needs to be taken into account (Equation 7.2).

In general, the fraction of the enzyme population that inactivates relatively fast (*i.e.* x_1) decreases with an increasing amount of molecular sieve powder. The inactivation rate constant k_1 increases with an increasing amount of molecular sieve powder. With a larger amount of molecular sieves, the equilibrium in water between the molecular sieves and Cov is thus reached faster, and the difference between A_0 and A_t, in the persistent part of residual activity of Cov, is smaller.

Table 7.1. Parameters of the single exponential decay function (Equation 7.2) used to fit the dehydration-induced inactivation of Cov with different amounts of molecular sieve powder.

mg molecular sieve powder / mg Cov	x_1	k_1 (h^{-1})
1.5	0.56	0.16
3	0.44	0.23
4	0.56	0.40
6	0.28	0.74
12	0.11	0.59

The dehydration-induced inactivation of Cov by molecular sieve powder thus seems to occur in three phases: (1) an initial, rapid, major dehydration-induced inactivation, of which the extent is dependent on the amount of molecular sieve powder, and that takes place during the first activity measurement (1 h), (2) a phase in which Cov inactivates with rate constant k_1 (20 h), and (3) a phase in which the activity of Cov remains practically constant. Due to practical reasons the rate of the initial major dehydration-induced inactivation could not be measured.

Previously it was found that the kinetics of the coupling can be described well with a two-substrate kinetic model with two inhibitory products,

$$v = v_{\max} \cdot \frac{[A]}{[A] + K_m^A \cdot \left(1 + \frac{[P]}{K_i^P} + \frac{[Q]}{K_i^Q}\right)} \cdot \frac{[B]}{[B] + K_m^B} \tag{7.3}$$

where v is the reaction rate (mmol L^{-1} h^{-1}), v_{max} is the maximum reaction rate (mmol L^{-1} h^{-1}), *[A]* and K_m^A, and *[B]* and K_m^B are the concentrations (mM) and the Michaelis constants (mM) of substrate A (Z-Phe-OCam) and of substrate B (Phe-NH_2) respectively, *[P]* is the concentration of glycolamide (mM), *[Q]* is the concentration of the dipeptide (mM), and K_i^P and K_i^Q are the respective inhibition constants (mM). The estimated kinetic parameters associated with the model are given in Table 7.2 (column 'Coupling kinetics only').

A new model was set up, by combining both the coupling kinetics (Equation 7.3) and the dehydration kinetics (Equation 7.2). As a result, the maximum rate v_{max} is formulated as a time-dependent quantity, due to the inactivation by dehydration,

$$v_{\max}(t) = [Cov] \cdot k \cdot (x_1 e^{-k_1 t} + (1 - x_1)) \tag{7.4}$$

where *[Cov]* is the concentration of Cov (g L^{-1}) and *k* is a fitting parameter for the initial dehydration-induced inactivation (mmol h^{-1} g^{-1}).

The combined model was used to fit the progress curve data of previous work (Vossenberg *et al.* Submitted-b), in which 1.5 mg molecular sieve powder per mg Cov was used in combination with various initial substrate and product concentrations, and the progress curve data of this work, in which 3, 4, 6, and 12 mg molecular sieve powder per mg Cov was used in combination with two different Z-Phe-OCam concentrations. In the kinetic models used in the previous study to fit progress curve data, dehydration-induced inactivation of Cov by the molecular sieve powder was ignored. It is, however, possible that the dehydration was hidden in the estimated kinetic parameter values.

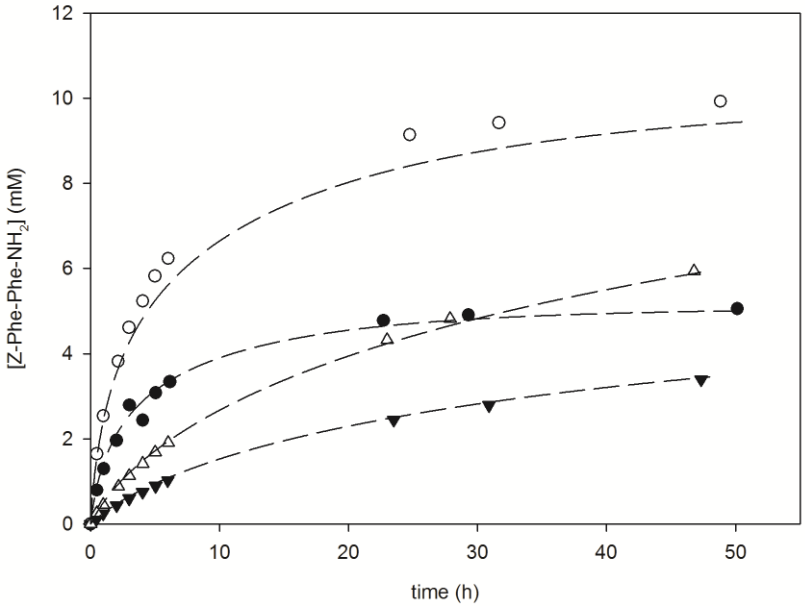


Figure 7.2. Experimental data with two different initial Z-Phe-OCam concentrations (5 •▼ and 10 ○Δ mM) and two different molecular sieve powder amounts (1.5 •○ and 12 ▼Δ mg powder per mg Cov), and fitted progress curves of Z-Phe-Phe-NH_2 synthesis catalyzed by Cov, using a two-substrate kinetic model with two inhibitory products and incorporating enzyme dehydration. Parameters of the single exponential decay function: Table 7.1; estimated kinetic parameter values: Table 7.2 (column 'Coupling and dehydration kinetics combined'); estimated $v_{max}(0)$ values: Figure 7.3.

All the progress curve data of this and previous work were fitted together to estimate values for k, K_m^A, K_m^B, K_i^P, and K_i^Q (selection of data shown in Figure 7.2). The values for x_1 and k_1 were taken from Table 7.1. Except for k, the parameter values were assumed to be independent of the amount of molecular sieve powder. The various values for k, and thus for $v_{max}(0)$, give an indication of how the initial dehydration-induced inactivation varies with the amount of molecular sieve powder. The parameter values from a fit with the combined model are given in Table 7.2 (column 'Coupling and dehydration kinetics combined'). The estimated values for $v_{max}(0)$ as a function of the amount of molecular sieve power are given in Figure 7.3.

The difference between the parameter values from a fit with Equation 7.3 (coupling kinetics only) and from a fit that combines coupling and dehydration is minimal (Table 7.2). The estimated kinetic parameters for K_m^A, K_m^B, K_i^P, and K_i^Q from previous work were thus hardly 'polluted' with the dehydration of Cov that took place during the reaction time course in the presence of 1.5 mg molecular sieve powder per mg Cov. The current estimated value for $v_{max}(0)$ is however significantly different from previous work (Table 7.2, Figure 7.3).

As expected, the $v_{max}(0)$ decreases with an increasing amount of molecular sieve powder (Figure 7.3). This decrease is quite significant, which means that to minimize the initial dehydration-induced inactivation of Cov, and therefore take advantage of the high initial activity of Cov, the amount of molecular sieve powder should be carefully selected, taking into account that a certain minimum amount molecular sieves are required to prevent hydrolysis of the activated substrate.

Table 7.2. Estimated kinetic parameters for Cov-catalyzed dipeptide synthesis in THF.

Parameters	Coupling kinetics only	Coupling and dehydration kinetics combined
K_m^A (mM)	2700	2700
K_m^B (mM)	3.1	3.1
v_{max}(mmol L^{-1} h^{-1})	1300	Figure 7.3
K_i^P (mM)	2.5	2.5
K_i^Q (mM)	8.9	8.0

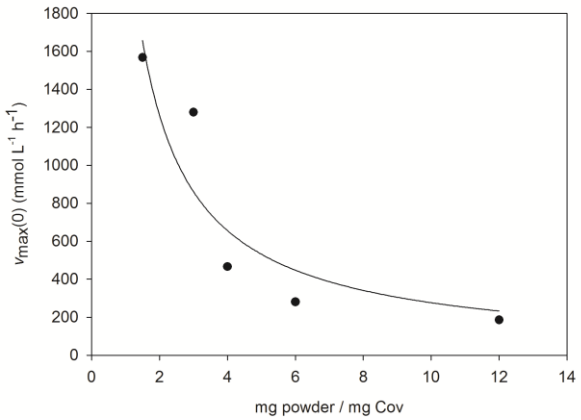


Figure 7.3. Estimated $v_{max}(0)$ values for different amounts of molecular sieve powder.

To validate the applicability of the two-substrate kinetic model with two inhibitory products and incorporating enzyme dehydration over longer periods of time, Cov was reused 5 times with 6 mg molecular sieve powder per mg Cov. The kinetic parameter values from Table 7.2 (column 'Coupling and dehydration kinetics combined') and Figure 7.3, the parameters of the single exponential decay function (Table 7.1), and the initial substrate concentrations, were used in the model to predict the independent experimental data. The model could predict the resulting experimental data quite well (Figure 7.4).

7.4 Conclusions

Dehydration-induced inactivation of Cov by molecular sieve powder was found to occur in three phases: (1) an initial, rapid, major dehydration-induced inactivation that takes place during the first activity measurement (1 h), (2) a phase of first-order inactivation (20 h), and (3) a phase in which the activity of Cov remains constant. The coupling of Z-Phe-OCam and Phe-NH_2 in THF catalyzed by Cov in the presence of different amounts of molecular sieve powder could be described and predicted well with a two-substrate kinetic model with inhibitory products and incorporating these Cov dehydration phases.

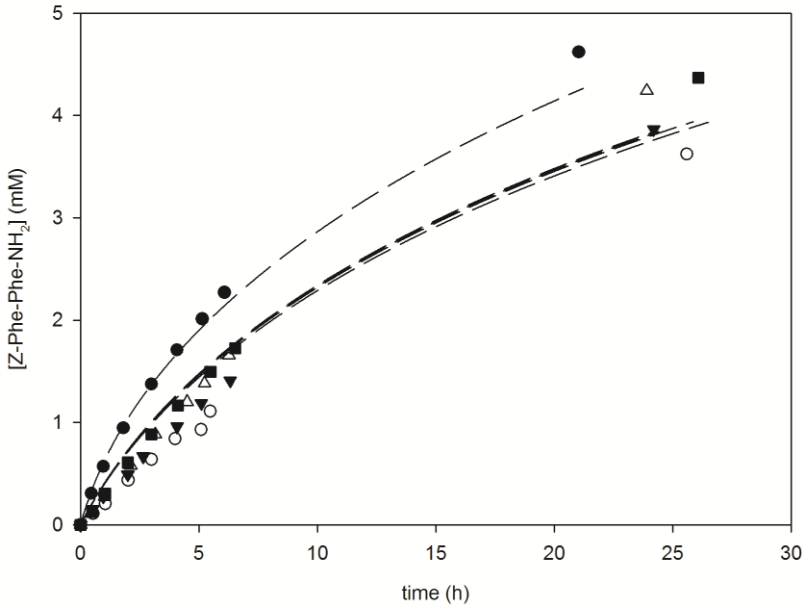


Figure 7.4. Experimental data of reuse (1•, 2○, 3▼, 4Δ, 5■) of Cov with 6 mg molecular sieve powder per mg Cov, and progress curves of Z-Phe-Phe-NH_2 synthesis catalyzed by Cov predicted using a two-substrate kinetic model with two inhibitory products and incorporating enzyme dehydration. Used parameter values: Table 7.1, Table 7.2 (column 'Coupling and dehydration kinetics combined'), and Figure 7.3.

CHAPTER 8

General discussion: Process design for chemo-enzymatic peptide synthesis in near-anhydrous organic media

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. Process design for enzymatic peptide synthesis in near-anhydrous organic media. Submitted.

Abstract

This work is a case study about a process design for enzymatic peptide synthesis, which is based on and inspired by previously established data about the Alcalase-catalyzed coupling of an amino acid amide and a chemically synthesized activated N-protected amino acid carbamoylmethyl ester in near-anhydrous tetrahydrofuran. The choices with regard to Alcalase formulation, type of reactor, way to control the water content, and whether or not to recycle the enzyme, are discussed. In addition, an estimate is given for the reactor size, volumes of solvent, amounts of substrate, enzyme and molecular sieves, needed in order to meet a specific demand for peptides. We believe that this case study gives a good impression of the various choices that have to be made when designing a process for enzymatic peptide synthesis and the implications of these choices.

Keywords: *process design, peptide synthesis, immobilized protease, enzyme reusability, molecular sieves, near-anhydrous*

8.1 Introduction

This work is a case study about a process design for enzymatic peptide synthesis, which is based on and inspired by previously established data about a certain peptide synthesis route. We have focused on the Alcalase-catalyzed coupling of an amino acid amide and a chemically synthesized activated N-protected amino acid carbamoylmethyl (Cam) ester in near-anhydrous tetrahydrofuran (THF) (Figure 8.1) (Miyazawa *et al.* 2002; Nuijens *et al.* 2010; Quaedflieg *et al.* 2010; Nuijens *et al.* 2011; Vossenberg *et al.* 2011; Vossenberg *et al.* 2012a; Vossenberg *et al.* 2012b). A carbamoylmethyl ester was used instead of the more regularly used methyl or ethyl esters because of its positive effect on the coupling rate (Miyazawa *et al.* 2002). THF was used as solvent because preliminary studies (data not shown) showed good results with respect to the solubility of the substrates and the stability and activity of the enzyme in THF. The choices with regard to Alcalase formulation, type of reactor, way to control the water content, and whether or not to recycle the enzyme, are discussed. In addition, an estimate is given for the reactor size, volumes of solvent, amounts of substrate, enzyme and molecular sieves, needed in order to reach a relevant specified demand for peptides. Furthermore, some possible solutions to bottlenecks of the process design, that is proposed in this work, are suggested.

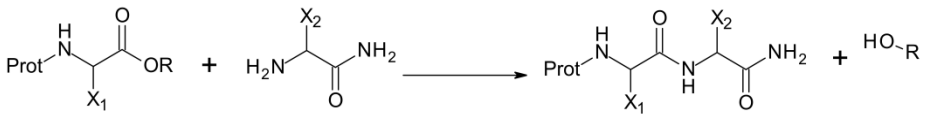


Figure 8.1. Coupling of an N-protected (protected using benzyloxycarbonyl), C-terminally activated amino acid (the activating group, R, is a carbamoylmethyl) with an amino acid amide to give a dipeptide and glycolamide. X_1 and X_2 can be any amino acid side chain.

We realize that the proposed process design may not be considered realistic for dipeptides, as these will not be synthesized on a large scale by enzymatic peptide synthesis, especially when using Cam-esters as substrate. Such enzymatic synthesis is more realistic for the enzymatic coupling of relatively large chemically synthesized peptide fragments (Nuijens *et al.* submitted). The synthesis of the

Cam-esters, which are relatively expensive in comparison to regular amino acids, only becomes feasible when coupling peptide fragments. There are examples (*i.e.* aspartame (L-aspartyl-L-phenylalanine methyl ester)), however, that also for production of small peptides enzymatic synthesis can be a successful and competitive technique (Guzmán *et al.* 2007; Yagasaki & Hashimoto 2008).

We do believe that the present case study gives a good impression of the various choices that have to be made when designing a process for enzymatic peptide synthesis and of the implications of these choices. Furthermore, the case study makes the bottlenecks of the process very explicit. Before going into details, different aspects are introduced to set the direction for the process design, including a framework for peptide synthesis, an economic analysis of peptide synthesis, and an impression of enzyme-catalyzed synthesis in neat organic solvents.

8.1.1 Framework for peptide synthesis

Peptides, in particular oligopeptides, play an important role in the fields of health care, nutrition and cosmetics (Vulfson 1993; Lintner & Peschard 2000; Guzmán *et al.* 2007; Vivó-Sesé & Pla 2007; Stevenson 2009; Quaedflieg *et al.* 2010). Especially pharmaceutical peptides have gained attention in recent years. In 2005, Frost & Sullivan valued the global therapeutic peptides market at €760 million per year (in-Pharma Technologist 2005). This interest is caused by the development of novel drug delivery technologies, which allow the effective delivery of peptides, and the applications of peptides in therapeutics against *e.g.* cancer, cardiovascular diseases, infections, and metabolic disorders (Sewald & Jakubke 2002b; Sewald & Jakubke 2002a; Lien & Lowman 2003; News-Medical.Net 2005; Guzmán *et al.* 2007). Advantages of peptides over other small molecules are a high biological activity, a high specificity (minimal unspecific binding), and a low toxicity (minimal accumulation in tissues) (Marx 2005; Pichereau & Allary 2005; Ayoub & Scheidegger 2006).

Chemical synthesis is currently the most mature technique for the synthesis of peptides that range in length from 5 to 80 amino acids (Andersson *et al.* 2000; Sewald & Jakubke 2002a; Guzmán *et al.* 2007; Thayer 2011). Such a length is a

typical size for therapeutic peptides (PharmPro 2009). Chemical synthesis is, however, expected to be more and more combined with enzyme-catalyzed synthesis, resulting in chemo-enzymatic approaches towards peptide synthesis (Hou *et al.* 2005; Guzmán *et al.* 2007). The racemization that hampers chemical synthesis can be prevented by forming the peptide bond enzymatically (Gill *et al.* 1996; Sewald & Jakubke 2002b; Kumar & Bhalla 2005).

Due to the ongoing developments in peptide synthesis and broadening of applications of peptides, competition in the field of peptide synthesis will increase. A process design for chemo-enzymatic peptide synthesis should therefore inevitably aim at minimal production costs. In the next paragraph we will analyze the bottleneck in the production costs.

8.1.2 Economic analysis of peptide synthesis

The global scale for oligopeptides, which currently are mainly chemically synthesized, is around 1 - 50 kg per year per peptide, where the selling price of peptides of about 10 amino acids in length is €10.000 - €50.000 per kg. This price increases radically with an increase in peptide size: a peptide consisting of 40 amino acids is sold for €1.000.000 - €2.500.000 per kg (IMS Health Database 2009, with production costs being estimated at 4 % of the selling price). These prices are due to the relatively small market scale, but also due to an increase in production costs with an increase in peptide size. The costs are to a large extent caused by downstream processing (DSP) as the difficulty of purification increases with peptide size, assuming chemical synthesis, due to increasing numbers of side reactions leading to side-products that are very similar to the desired product (Nuijens *et al.* 2012). The production costs per kg of peptide increases exponentially with increase in peptide length (Figure 8.2). At a certain length of peptide, however, chemical synthesis has to compete with fermentation, with respect to synthesizing profitable oligopeptides. Short oligopeptides are difficult to produce with fermentation due to difficulties in creating successful expression systems and in purifying the peptide product from the fermentation broth. In addition, the peptide products may be rapidly degraded by proteases, be unstable or toxic to cells (Smith *et al.* 1992; Sewald & Jakubke 2002b).

Compared to the selling price of the oligopeptides, the costs of substrates (*i.e.* amino acids) are minimal. Regular amino acids cost €10 - €50 per kg at a ton scale. N-protected and C-activated amino acids are somewhat more expensive: €100 - €500 per kg at a ton scale. If a chemo-enzymatic approach is used to synthesize peptides, the enzyme costs also need to be considered. Alcalase covalently immobilized onto macroporous acrylic beads (in this paper abbreviated as Cov), for example, is €800 per kg at a 10-kg scale (ChiralVision 2012). This enzyme was used in previous work to synthesize peptides (Vossenberg *et al.* 2012b; Vossenberg *et al.* submitted-b; Vossenberg *et al.* submitted-c). These enzyme costs thus also seem rather minimal compared to the selling price of the oligopeptides. Nevertheless, the total enzyme costs will of course depend on how much enzyme is needed, which is determined by the kinetics of the coupling reaction in combination with the stability of the enzyme.

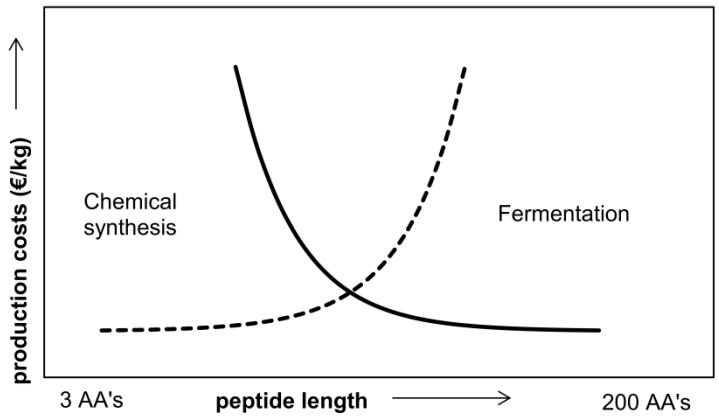


Figure 8.2. Illustrative example of how the production costs of a peptide varies with its length, both for chemical synthesis (dashed line) and fermentation (solid line).

8.1.3 Towards a process design for chemo-enzymatic peptide synthesis

Based on the above cost analysis and regarding the remaining costs, it is expected that DSP costs are the most important. A process design for peptide synthesis should, therefore, aim at minimizing the DSP costs. To achieve this, one can either focus on the DSP itself or on the reaction(s) that precede DSP. For example, if the result of a certain reaction is only one product, the DSP costs will be rather

minimal. In this study, the focus is therefore on the optimization of the reaction(s) that precede DSP. To avoid racemization, the coupling of the amino acids is enzymatically catalyzed. To prevent hydrolytic side reactions, dry conditions should be maintained in our system. The aim is close to full substrate conversion to the relevant peptide. In addition, the product should be as concentrated as possible in order to simplify product recovery and purification, as well as minimize the required reactor volume and waste stream volumes (Straathof *et al.* 2002).

8.1.4 Enzyme-catalyzed synthesis in neat organic solvents

According to Straathof *et al.* there are currently few industrial biotransformations being carried out in a neat organic solvent (Straathof *et al.* 2002); the ones that are, involve (trans)esterification or amidation reactions. One of the largest applications of enzymes in non-aqueous media is the lipase-catalyzed modification of oils and fats, for example in the production of margarine fats and in the manufacture of cocoa butter substitutes (Halling 1990; Vulfson 1993; Sheldon 1996). There are different reasons (Adlercreutz 1996; Sheldon 1996; Yang & Russell 1996; Khmelnitsky & Rich 1999; Schmid *et al.* 2001; Bordusa 2002; Hayes 2002; Castro & Knubovets 2003; Sanchez & Demain 2010; Yu *et al.* 2010) for executing enzyme-catalyzed reactions in non-aqueous media, such as:

- prevention of hydrolytic side-reactions,
- reversal of hydrolytic reactions,
- increase of substrate solubility,
- improving substrate specificity and enzyme enantioselectivity,
- prevention of microbial contamination,
- facilitation of enzyme recovery.

In non-aqueous media, it is important to control the water content, or more specifically the water activity, of the solvent. The solvent should not be completely dry as enzymes need some water to maintain their catalytic activity (Gaertner & Puigserver 1989; Adlercreutz 1991; Klibanov 2001; Bordusa 2002; Halling 2004). Controlling the water content becomes more challenging if the water activity changes due to water formation during the reaction. Various methods have been proposed for the removal of water, such as water exchange with saturated salt

solutions or with a gas phase with specific humidity, vacuum or azeotropic distillation, pervaporation, or the use of molecular sieves Cassells & Halling 1988; Ergan *et al.* 1990; Khan *et al.* 1990; Kvittingen *et al.* 1992; Van der Padt *et al.* 1993; Wehtje *et al.* 1993; Svensson *et al.* 1994; Kwon *et al.* 1995; Rosell *et al.* 1996; Jeong & Lee 1997; Ujang *et al.* 1997; Wehtje *et al.* 1997; Gubicza *et al.* 2000; Shick Rhee *et al.* 2001; Won & Lee 2001; Hayes 2002; Xu 2003; Kapoor & Gupta 2012).

In the coupling reaction that we studied, the coupling of an amino acid amide and a chemically synthesized activated N-protected amino acid Cam-ester, water is not formed: the reaction products are a dipeptide and glycolamide (Figure 8.1). A continuous removal of water is therefore, in principle, not necessary. Nevertheless, we did find that a carefully chosen amount of molecular sieves is required to prevent hydrolysis of the activated substrate (in the present system only the hydrolysis of substrate, and not of the product, occurs) (Vossenberg *et al.* 2012a). The water is introduced into the system by the enzyme formulation, which serves as a water carrier. Cross-linked enzyme aggregates (CLEA) containing Alcalase, Alcalase immobilized onto dicalite, and Cov, for example, need to be hydrated, prior to use, for the enzyme formulation to become an active catalyst for dipeptide synthesis in near-anhydrous organic media (Vossenberg *et al.* 2012b).

Protease-catalyzed peptide synthesis in monophasic organic solvents has also been studied by other research groups with a range of proteases: chymotrypsin (Gaertner & Puigserver 1989; Clapés *et al.* 1990; Kimura *et al.* 1990; Kise & Hayakawa 1991; Clapés *et al.* 1992; Richards *et al.* 1993; Rolland-Fulcrand *et al.* 1994; Jönsson *et al.* 1995; Capellas *et al.* 1996; Sergeeva *et al.* 1997; Vasic-Racki *et al.* 2003), subtilisin Carlsberg (Ferjancic *et al.* 1990; Sergeeva *et al.* 1997; Klein *et al.* 2000; Okazaki *et al.* 2000; Miyazawa *et al.* 2002), subtilisin BPN' (Kise & Hayakawa 1991), subtilisin 72 (Belyaeva *et al.* 2005), thermolysin (Oyama *et al.* 1981; Kimura *et al.* 1990; Nakanishi *et al.* 1990; Filippova & Lysogorskaya 2003), Alcalase (Chen *et al.* 1992; Hou *et al.* 2006; Li *et al.* 2008; Nuijens *et al.* 2010a), and papain (Zhang *et al.* 1996). Although there is a lot of research data available about peptide synthesis with various proteases, we studied in detail different aspects of a specific peptide synthesis route, which, when combined, allow us to do this case study. These different aspects include investigation of the:

- optimal conditions for synthesis of dipeptides in organic media (Vossenberg *et al.* 2012a),
- performance of Alcalase formulations in near-dry organic media (Vossenberg *et al.* 2012b),
- long-term stability and reusability of immobilized Alcalase with respect to dipeptide synthesis (Vossenberg *et al.* submitted-c),
- kinetics of the dipeptide synthesis (Vossenberg *et al.* submitted-b),
- effect of enzyme dehydration on the dipeptide synthesis kinetics (Vossenberg *et al.* submitted-a).

8.2 Process design for chemo-enzymatic peptide synthesis

In order to design a process for enzymatic peptide synthesis various choices have to be made. Below choices and their implications with regard to enzyme formulation, design criteria, type of reactor, way to control the water content, and whether or not to recycle the enzyme, are discussed, leading to a process design. In addition, possible solutions to bottlenecks in the process design are discussed.

8.2.1 Choice of enzyme formulation

Previously we found Cov to be the most promising enzyme formulation for large-scale dipeptide synthesis in organic media (Vossenberg *et al.* 2012b). This formulation has a reasonable activity with respect to dipeptide synthesis in near-anhydrous organic media, and, from a practical point of view, a reasonable size (150 – 300 μm in diameter (ChiralVision 2011)), and a uniform shape, which is an attractive feature for the purpose of modeling and its use in a packed-bed. Small, deformable, and irregularly shaped particles can result in channelling, large pressure drops, and clogging in a packed-bed (Van 't Riet & Tramper 1991; Xu 2003; Buchholz *et al.* 2005). Furthermore, we observed that Cov is stable in THF, with and without molecular sieve powder. Nevertheless, with molecular sieve beads, in rotating reaction vials, Cov lost activity over time due to mechanical forces between Cov and the molecular sieve beads (Vossenberg *et al.* submitted-c). It is, however, probable that this mechanical damage does not occur in a packed-

bed or stirred reaction vessel, as the particles will not tumble over one another along a wall.

8.2.2 Design criterion

Based on the previously mentioned scale for oligopeptides, the design criterion of our process is to produce 50 kg of peptide per year (section '8.1.2 Economic analysis of peptide synthesis'). Depending on the size of a company, the timeframe in which this amount of peptide should be produced will differ. We, therefore, propose a process design for two types of companies:

1) a large company (*i.e.* a company that has a large product portfolio of which the products are synthesized in a multi-purpose reactor) that wants to produce 50 kg of peptide in a single run in a relatively short timeframe, *i.e.* two weeks,
2) a small company (*i.e.* a dedicated company that has a reactor dedicated to a specific product) that wants to produce 50 kg of peptide in 6 months.

The above mentioned timeframes are reactor operation times, excluding downtime of the reactor.

In this study the coupling of the amino acids is enzymatically catalyzed (Figure 8.1). We set the target at 99 % of substrate conversion, in order to have a high product to substrate ratio at the end of the run and thereby facilitate DSP. When this target is further increased, the enzyme concentration needed to reach the new target in the same amount of time increases dramatically (Figure 8.3). Figure 8.3 is a prediction based on the reaction kinetics of the Cov-catalyzed coupling of phenylalanine amide (Phe-NH_2) and the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam) in THF (Vossenberg *et al.* submitted-b; Vossenberg *et al.* submitted-a), in a batch reactor.

Previously (Vossenberg *et al.* submitted-b) it was found that the reaction kinetics of the coupling can be described well with a two-substrate kinetic model with two inhibitory products,

$$v = k_{cat} \cdot [Cov] \cdot \frac{[A]}{[A] + K_m^A \cdot \left(1 + \frac{[P]}{K_i^P} + \frac{[Q]}{K_i^Q}\right)} \cdot \frac{[B]}{[B] + K_m^B} \tag{8.1}$$

where v is the reaction rate (mmol L^{-1} h^{-1}), k_{cat} is the catalytic rate constant (mmol h^{-1} g^{-1}), *[Cov]* is the concentration of Cov (g L^{-1}), *[A]* and K_m^A, and *[B]* and K_m^B are the concentrations (mM) and the Michaelis constants (mM) of substrate A (Z-Phe-OCam) and of substrate B (Phe-NH_2) respectively, *[P]* is the concentration of glycolamide (mM), *[Q]* is the concentration of the dipeptide (mM), and K_i^P and K_i^Q are the respective inhibition constants (mM). The estimated values for the kinetic parameters are given in Table 8.1, where the value of k_{cat} is based on the synthesis in the presence of 1.5 mg molecular sieve powder per mg Cov. The kinetic characteristics were determined from experiments in which the coupling reaction was followed up to full conversion at various initial concentrations of substrate and product.

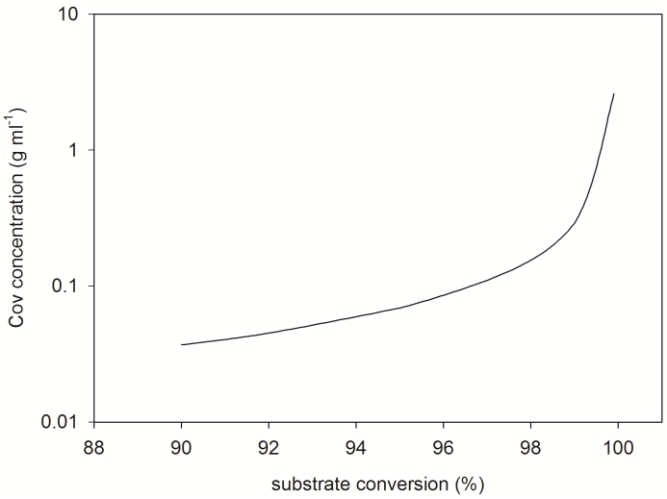


Figure 8.3. Illustrative example of how the enzyme concentration needed varies with the substrate conversion target. The time for the conversion is assumed constant (*i.e.* 24 h). The figure is based on the reaction kinetics of the Cov-catalyzed coupling of Phe-NH_2 (15 mM) and Z-Phe-OCam (15 mM) in THF in a batch reactor with 1.5 mg molecular sieve powder per mg Cov.

To keep the product stream as clean as possible, a 1:1 molar ratio of the starting concentrations of both amino acids is used in the coupling reaction of the case

study. The amino acids react stoichiometrically, *i.e.* in a 1:1 molar ratio (Figure 8.1). Thus, at a 100 % conversion to products, both substrates are completely depleted. Nevertheless, based on the reaction kinetics of the Cov-catalyzed coupling of Phe-NH_2 and Z-Phe-OCam, it would be advantageous, with respect to the enzyme concentration, to use an excess amount of either Phe-NH_2 or Z-Phe-OCam, as both substrates are rate limiting (Figure 8.4). Using an excess amount of an amino acid will, however, not be an option as it makes DSP much more complicated.

Table 8.1. Estimated kinetic parameters for Cov-catalyzed dipeptide synthesis in THF, in the presence of 1.5 mg molecular sieve powder per mg Cov.

Parameters	Values
K_m^A (mM)	2700
K_m^B (mM)	3.1
k_{cat} (mmol h^{-1} g^{-1})	141
K_i^P (mM)	2.5
K_i^Q (mM)	8.0

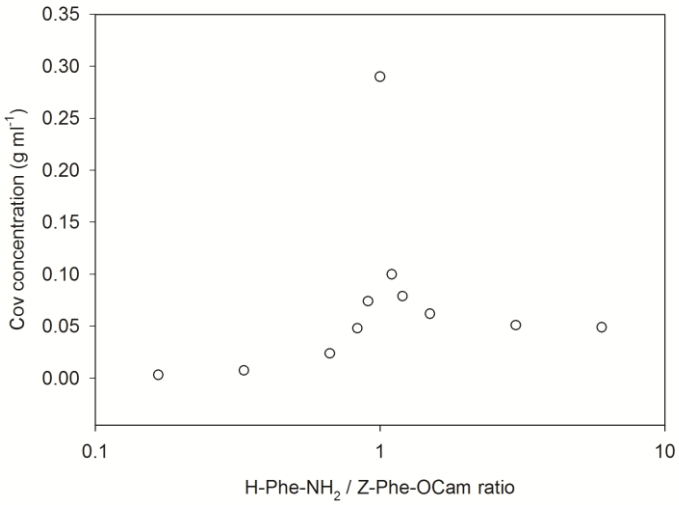


Figure 8.4. Illustrative example of how the enzyme concentration needed varies with the initial substrate ratio. The time for the conversion (99 % conversion of the limiting substrate) is constant (*i.e.* 24 h). The figure is based on the reaction kinetics of the Cov-catalyzed coupling of Phe-NH_2 (15 - 90 mM) and Z-Phe-OCam (15 - 90 mM) in THF in a batch reactor with 1.5 mg molecular sieve powder per mg Cov.

As mentioned before (section '8.1.3 Towards a process design for enzymatic peptide synthesis'), the product stream should, in view of DSP costs, be as concentrated as possible. The solubility of Z-Phe-Phe-NH_2, the dipeptide on which this design is based, is about 16 mM in THF (Vossenberg *et al.* submitted-b). To prevent product precipitation in order to minimize practical complications, we aim at a final product concentration of 15 mM.

8.2.3 Type of reactor

There are three main reactor types that could be used for the enzymatic coupling of the amino acids: (1) a batch reactor, (2) a continuous stirred-tank reactor (CSTR), or (3) a packed bed reactor as plug flow reactor (PFR). For this process a single CSTR will not be feasible due to kinetic considerations (Bischoff 1966; De Gooijer *et al.* 1996; Illanes & Altamirano 2008): Equation 8.1. In addition, we aim at a high amount of substrate conversion, which makes the CSTR even worse.

In industrial biotransformations, batch reactors are more popular than continuous reactors such as PFRs, especially for applications on a relatively small product scale (Straathof *et al.* 2002). A production scale of 50 kg of peptide per year, which is the aim of this study, is such a small-scale biotransformation. One of the reasons for this preference is that a batch reactor can be more easily used for different products (*i.e.* multi-product operation) than a PFR. In addition, the capital costs of a batch reactor are usually lower than that of a PFR. For large-scale biotransformations, however, a PFR will be preferred over a batch reactor due to the necessary downtime of the batch reactor (Balcão *et al.* 1996). For this study the use of a stirred batch reactor is thus preferred for the enzymatic coupling of the amino acids.

As we aim at a final product concentration of 15 mM, in order to prevent product precipitation, the volume of THF needed to produce 50 kg of peptide in a batch reactor is 7.5 m^3. A large company (Process design 1) thus needs a reactor with a working volume of > 7.5 m^3 (THF + Cov + molecular sieves) to produce 50 kg of peptide in one batch of two weeks. For a small company that wants to produce 50 kg of peptide in 6 months (Process design 2), the reactor volume is at this point still a variable depending on the specific process design. It is highly unlikely that

this relatively small amount of peptide will be produced in a single run of 6 months, due to the risk of losing the complete batch. The total amount of product will therefore be produced in multiple runs. The process will be divided into several batches, each produced in the same reactor, spanning a certain timeframe. The timeframe will be set identical for each batch. The choice for a certain batch time is discussed below. The small company will need a smaller reactor volume than the large company as the total amount of aimed product remains the same for both the small and large company, but the production is divided into several batches for the small company. The total amount of solvent does, however, not change as this is determined by the solubility of the product. We do realize that the manpower needed increases as the number of batches increases.

8.2.4 Implications of the use of molecular sieves

Cov needs to be hydrated prior to use for the enzyme formulation to become an active catalyst for peptide synthesis in near-anhydrous organic media (Vossenberg *et al.* 2012b). Due to this hydration, water is introduced into system. The addition of molecular sieves can prevent this water from reacting with the activated substrate and thus maximize the synthesis to hydrolysis ratio (*i.e.* the S/H ratio) of the process. Preventing hydrolytic side reactions will reduce DSP costs. Nevertheless, an excess amount of molecular sieves or the use of molecular sieves over longer periods of time, dehydrate the enzyme and thereby inactivate it to a certain extent (Vossenberg *et al.* 2012b).

We found dehydration-induced inactivation of Cov by molecular sieve powder to occur, after an initial, almost instantaneous dehydration-induced inactivation, in two phases: (1) a phase of first-order inactivation (20 h) and (2) a relatively low plateau phase in activity (Vossenberg *et al.* submitted-a). The rate of dehydration in the phases increases with the amount of molecular sieves. To minimize the dehydration-induced inactivation of Cov, the amount of molecular sieve powder should be carefully selected, taking into account that a certain minimum amount of molecular sieves is required to prevent hydrolysis of the activated substrate. This dehydration-induced inactivation of Cov can, however, be counteracted by

intermediate rehydration (Vossenberg *et al.* 2012b). Dehydration-induced inactivation is thus a reversible process.

One can choose between molecular sieve beads or molecular sieve powder as a tool to prevent hydrolytic side reactions. Molecular sieve beads are more easy to separate from Cov than molecular sieve powder (Vossenberg *et al.* submitted-c). This separation is required in case of recycling and rehydrating Cov, and regenerating the molecular sieves. Nevertheless, when Cov was incubated with molecular sieve beads in THF in rotating reaction vials, Cov lost activity over time (Vossenberg *et al.* submitted-c). Mechanical damage of Cov by the molecular sieve beads was found to be the main reason for the instability of Cov. When Cov was incubated in THF with or without molecular sieve powder, it hardly inactivated within 30 days of incubation. In a batch reactor with a stirrer this mechanical damage of Cov by the molecular sieve beads will probably be minimal as the particles will not tumble over one another along a wall and they can easily follow the flow of the liquid medium and thus experience little shear. Molecular sieve beads are, however, bigger and denser than the Cov particles and thus more liable to be damaged by the stirrer of the batch reactor and have thus a higher chance of falling apart. If this happens the advantage of using molecular sieve beads due to their easy separation from Cov is no longer valid. For this study, therefore, the use of molecular sieve powder is preferred, at a concentration of 1.5 kg of molecular sieve powder per kg of Cov. This amount of molecular sieve powder prevents hydrolysis of the activated substrate (in case of Z-Phe-OCam) and causes a minimum amount of dehydration-induced inactivation (Vossenberg *et al.* submitted-b; Vossenberg *et al.* submitted-a).

8.2.5 Recycling

A large company (Process design 1) that wants to produce 50 kg of peptide in one batch will very likely not recycle the enzyme and store it for a year; they will use new enzyme for each batch and discard the used enzyme. For a small company (Process design 2), however, there are three options for Cov use in a batch reactor with molecular sieve powder:

1) use Cov once, *i.e*. use fresh, hydrated enzyme for each batch and discard the used enzyme,
2) reuse Cov with intermediate rehydration; by doing this, the dehydration-induced inactivation of Cov is reversed,
3) reuse Cov without intermediate rehydration, and accept the inevitable dehydration-induced inactivation of Cov to a final, relatively low value.

Each option has its advantages and disadvantages, which are summarized in Table 8.2 and discussed below.

The advantage of using fresh enzyme for each batch (Option 1) is that one can make use of the high initial activity of Cov, *i.e.* when it is not yet extensively dehydrated by the molecular sieves (Vossenberg *et al.* submitted-a). This option could, however, lead to significant enzyme costs as the enzyme is not recycled, although this significance is of course dependent on the selling price and thus length of the synthesized peptide. In addition, it will generate a lot of waste. This waste will not only consist of Cov, which needs to be dispensed after each batch, but also of the washing water and THF that are used in the hydration step in order for Cov to become an active catalyst (Vossenberg *et al.* 2012b). Previously, 1 ml of each washing liquid was used per 20 - 40 mg of Cov. The amount of Cov that can be washed with 1 ml of washing liquid could possibly be increased up to about 100 mg, which encompasses about 10 % of the washing liquid (assuming 1 kg of Cov to have a volume of about 1 L, as Cov has a density of 3.75 L kg^{-1} and a pore volume of 2.75 L kg^{-1} (information obtained from supplier)). This would mean that less liquid is needed to wash Cov, thus decreasing the waste stream.

The advantage of reusing Cov with intermediate rehydration for different batches (Option 2) is that one can make use of the high initial activity of Cov as well as minimize enzyme costs. Nevertheless, the same amount of washing liquid waste is generated for each batch as when using new enzyme. Furthermore, Cov and the molecular sieve powder need to be separated before Cov is rehydrated, which will be quite cumbersome due to the fine nature of the molecular sieve powder. Reusing Cov with intermediate rehydration does not seem a viable option, especially due to the required separation of Cov and the molecular sieve powder and the large amounts of washing liquid waste.

Table 8.2. Advantages and disadvantages of three options for the use of Cov.

Option	Advantage	Disadvantage
1) using new Cov for each batch	• high activity	• enzyme costs • waste (Cov + washing liquids)
2) reusing Cov with intermediate rehydration	• high activity • minimal enzyme costs	• waste (washing liquids) • separation of Cov and molecular sieve powder needed
3) reusing Cov without intermediate rehydration	• minimal enzyme costs • minimal waste	• low residual activity

The advantage of reusing Cov without intermediate rehydration (Option 3) for different batches is that enzyme costs and the amount of waste are minimized. It takes about 20 hours before freshly hydrated Cov reaches a plateau phase in activity at about 45 % of the initial activity, when using 1.5 kg molecular sieve powder per kg of Cov (Vossenberg *et al.* submitted-a). The advantage of using new enzyme for each batch compared to reusing Cov without intermediate rehydration, assuming no loss of Cov when recycled, can therefore only be exploited, if at all, when if a batch takes no longer than 20 hours, which can be seen in the difference between the points in Figure 8.5 for batch times of 1 day or less.

The plateau phase in activity, seen when we investigated the dehydration-induced inactivation of Cov by molecular sieve powder, suggests that an 'endless' use of Cov is possible. In practice this will probably not be true due to the continuous use of Cov. We, therefore, assumed a slow inactivation rate of Cov (*i.e.* $1.5 \times 10^{-4}\ h^{-1}$), which is based on previous work in which we observed a residual aqueous Cov activity of 90 % after 30 days of incubation in THF (Vossenberg *et al.* submitted-c). In addition, there will always be an amount of Cov that is lost when recycling it. Nevertheless, even if one assumes a 20 % loss of Cov per recycling step, the total amount of Cov needed to achieve ≥ 99 % of substrate conversion in each batch, is less than when using new enzyme for each batch (Figure 8.6). The calculations for Figure 8.6 assume that the loss of Cov is compensated for after each batch by adding new Cov to the reactor.

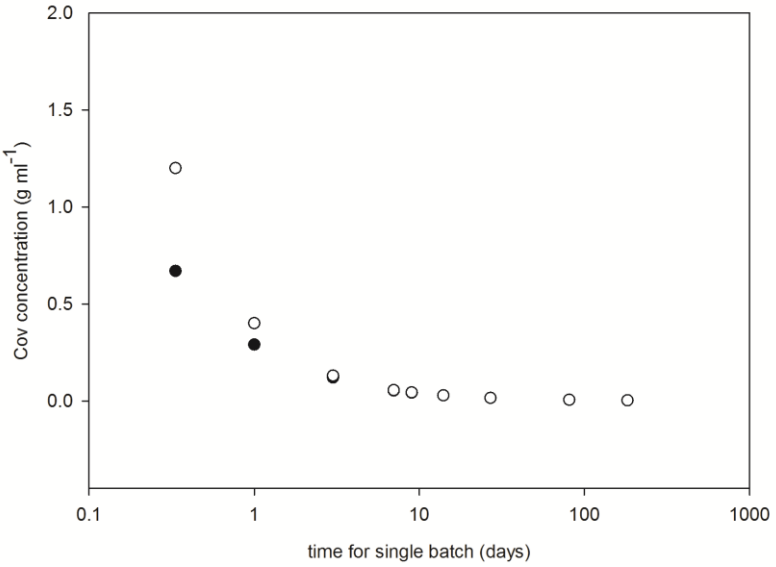


Figure 8.5. Cov concentration needed to reach ≥ 99 % substrate conversion in a certain batch time, using new enzyme for each batch (•) and reusing Cov without intermediate rehydration assuming no (○) loss of Cov per recycling step for a total reactor operation time of 6 months per year.

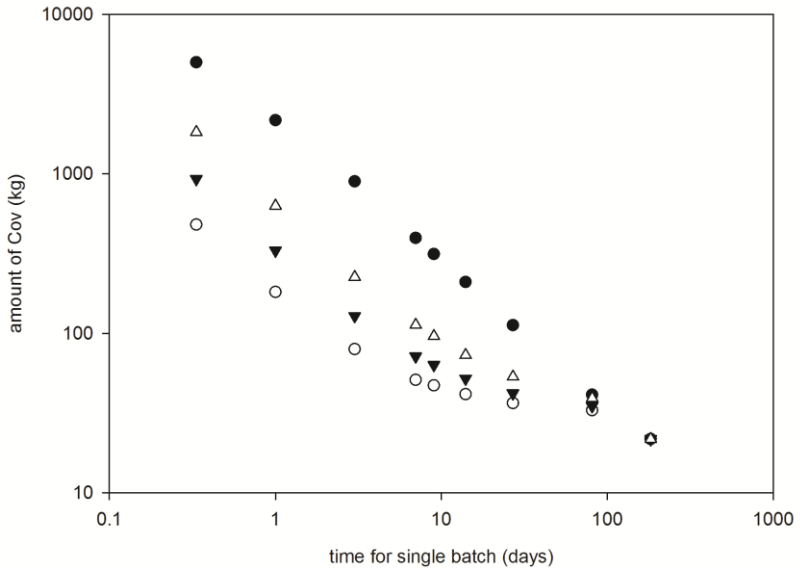


Figure 8.6. Total amount of Cov needed to reach ≥ 99 % substrate conversion in a certain batch time, using new enzyme for each batch (•), and reusing Cov without intermediate rehydration assuming 5 % (○), 10 % (▼), and 20 % (Δ) loss of Cov per recycling step for a total reactor operation time of 6 months per year.

When choosing an appropriate time for a single batch, the volume taken up by Cov should be considered. The percentage Cov volume of the total reactor volume should not be much more than 10 %, in order for the stirrer to mix the contents of the batch reactor properly and to minimize the product loss due to remaining product in Cov. Based on this, the time for a single batch should be ≥ 9 days, when reusing Cov, without intermediate rehydration assuming 5 % loss of Cov per recycling step (Figure 8.7).

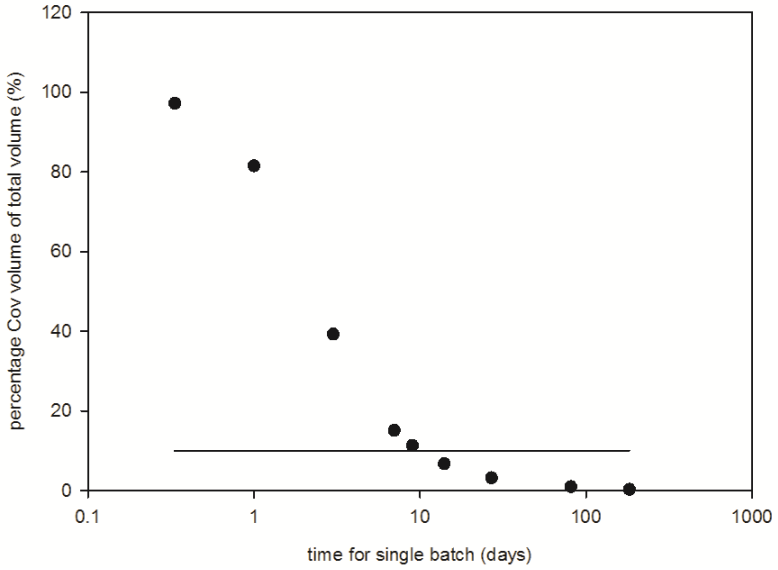


Figure 8.7. Percentage Cov volume of total reactor volume for a certain batch time, reusing Cov without intermediate rehydration assuming 5 % loss of Cov per recycling step for a total reactor operation time of 6 months per year.

For Process design 2 in this study, we suggest to reuse Cov without intermediate rehydration. In addition, we suggest to take 14 days for a single batch, which would imply the use of 41 kg Cov in total, which is 5x less than the total amount needed when using new enzyme for each batch (Figure 8.6), and thus 7 % volume taken up by Cov (Figure 8.7).

Figures 8.5 – 8.7 are based on the reaction kinetics of the Cov-catalyzed dipeptide synthesis (*i.e.* on Equation 8.1 and the corresponding kinetic parameters given in Table 8.1). When using new enzyme for each batch, the phase of first-order inactivation, due to dehydration by the molecular sieves, was incorporated in the

equation. In previous work we found that in the presence of 1.5 mg molecular sieve per kg Cov, 55 % of Cov inactivates with a rate constant of 0.16 h^{-1} (Vossenberg *et al.* submitted-a). This inactivation is due to dehydration. When reusing Cov without intermediate rehydration, this phase of first-order inactivation was not incorporated and thus the activity of Cov was set at 45 % of the initial activity. For both cases we incorporated a slow inactivation rate of Cov (*i.e.* 1.5×10^{-4} h^{-1}) due to its operational use. Using the reaction kinetics model and the inactivation rate of Cov, mass balances for the different components in the system were set up. The mass balances were numerically solved using the Euler method with a time step of 30 seconds.

8.2.6 Process design

Two process designs could be made (Figure 8.8 and 8.9), based on the aims and decisions made for this study:

- production of 50 kg of peptide per year,
- single batch time of 14 days,
- total reactor operation time per year:
    - 2 weeks (Process design 1)
    - 6 months (Process design 2), thus 13 batches per year,
- 99 % of substrate conversion,
- 1:1 molar ratio of the starting concentrations of both substrates,
- final product concentration of 15 mM,
- Cov as catalyst:
    - using Cov once (Process design 1)
    - reusing Cov without intermediate rehydration (Process design 2),
- batch reactor with molecular sieve powder.

In the first step of Process design 1 (Figure 8.8), Cov (210 kg in total) is hydrated so it will be an active catalyst for peptide synthesis. The hydration step consists of washing with water. Subsequently, Cov is washed twice with anhydrous THF, to remove the excess water. After washing, the THF can be recycled. During the recycle water should be removed from the THF by *e.g.* distillation or addition of

molecular sieves. After Cov hydration, the synthesis can start. The production of 50 kg of peptide at a final product concentration of 15 mM, occurs in one batch of 14 days. This means that 7.5 m^3 of THF is needed. The reactor working volume was set to 8 m^3 to encompass the THF, Cov, and the molecular sieves.

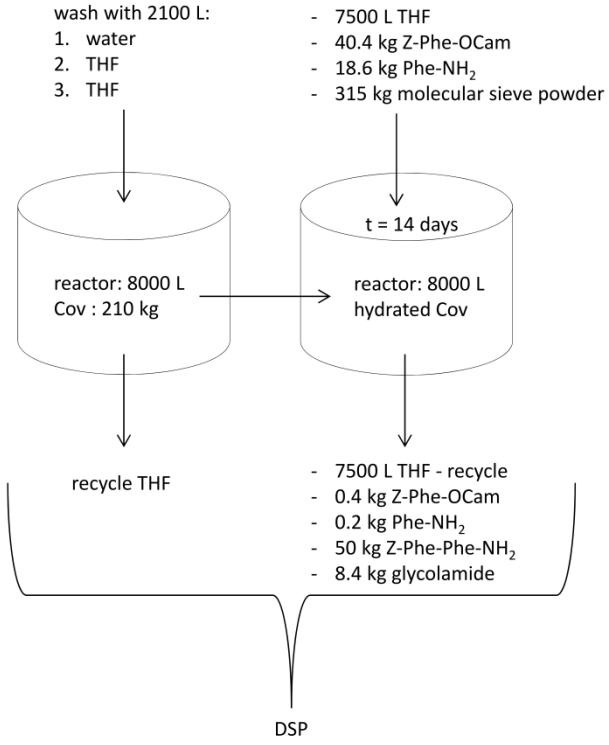


Figure 8.8. Process design 1 for coupling of Z-Phe-OCam and Phe-NH_2 with Cov: one batch of 14 days without enzyme recycle.

In Process design 2 (Figure 8.9), Cov (16 kg initially; 41 kg in total) is also first hydrated. The amount of solvents in the hydration step is smaller compared to Process design 1, as less Cov needs to be used. The production of 50 kg of peptide at a final product concentration of 15 mM, is divided into 13 batches of 14 days each. This means that 570 L of THF per batch is needed. The reactor working volume was set to 650 L to encompass the THF, Cov, and the molecular sieves. For each synthesis batch, new substrates, Cov and molecular sieves have to be added. The addition of new Cov is needed to compensate for the 5 % of recycling loss and the inactivation of Cov in time. As new Cov is added, also additional molecular

sieves have to be added in order to keep the molecular sieve powder to Cov ratio constant.

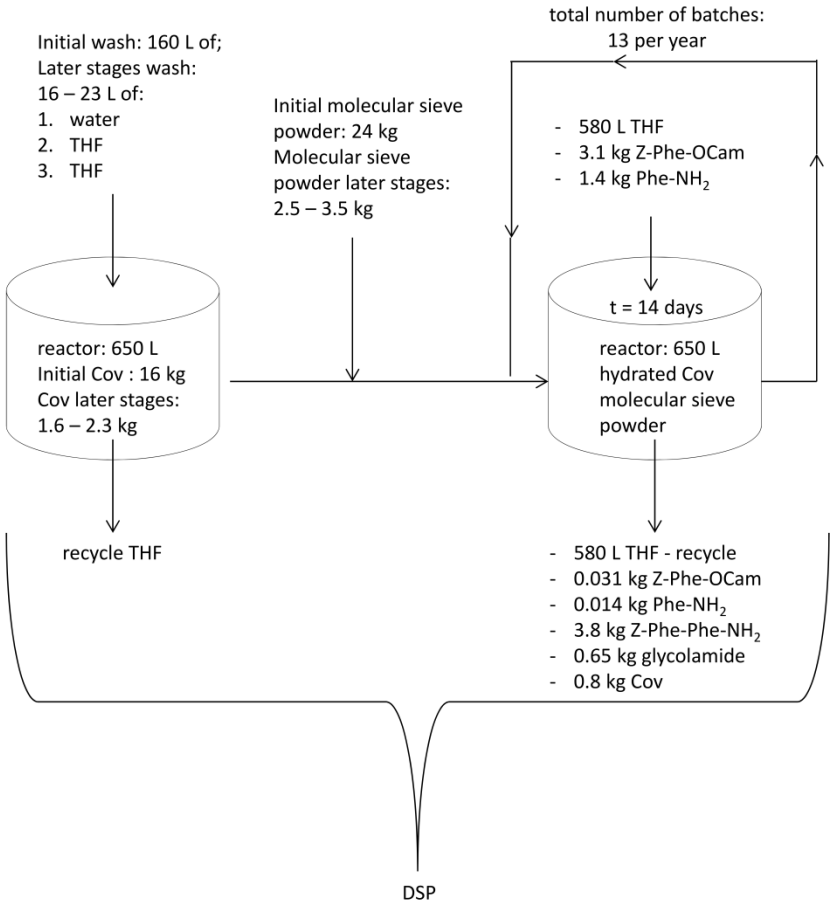


Figure 8.9. Process design 2 for coupling of Z-Phe-OCam and Phe-NH_2 with Cov: 13 batches of 14 days with enzyme recycle.

We believe that the reactor sizes in both process designs are quite reasonable as, for example, The PolyPeptide Group chemically synthesizes up to 100 kg of peptide in reactors up to 12 m^3 (The PolyPeptide Group 2012). It seems reasonable that also large companies will have (depreciated) reactors of 8 m^3 in size that can be used for Process design 1. The manpower needed in Process design 2 is larger than in Process design 1, but for small companies the risk of losing a batch, if the peptide is produced in one batch, is probably too large.

The water activities that we work with in the proposed enzymatic peptide synthesis are close to zero and thus on-line monitoring of the water activity will probably be infeasible. The only way water is introduced in the system is through the hydrated enzyme formulation. By adding molecular sieves we aim to prevent this water from reacting with the activated substrate and thus maximize the synthesis to hydrolysis ratio (*i.e.* the S/H ratio) of the process. Samples could be taken from the reactor to check whether the hydrolysis of the substrate is indeed absent. If not, more molecular sieves should be added during a batch.

For Process design 1 one needs to spend €168.000 (assuming Cov to be €800 per kg (ChiralVision 2012)) on the enzyme formulation to make 50 kg of peptide, thus €3.360 per kg of peptide. This is quite a large amount if compared to the selling price of peptides of about 10 amino acids in length (*i.e.* €10.000 - €50.000 per kg). Nevertheless, when synthesizing a peptide consisting of 40 amino acids, which can be sold for €1.000.000 - €2.500.000 per kg, these enzyme costs may be feasible. For Process design 2, 41 kg of Cov is needed in total. One thus needs to spend €32.800 on the enzyme formulation to make 50 kg of peptide, thus €655 per kg of peptide. These costs seem feasible even when synthesizing a peptide consisting of 10 amino acids.

For both designs, there are of course many other costs to consider besides enzyme costs, in order to decide whether the process designs are industrially viable. The large amounts of THF that are needed for both process designs (*i.e.* 9600 L for Process design 1 and 7910 L for Process design 2) can lead to high solvent costs (*i.e.* €14.000 - €17.000 assuming €2 per kg of THF). This is one of the reasons that we propose to recycle the THF. Such recycling of THF will also minimize the environmental impact of the process. Another cost factor are the costs for the peptide fragments (fragment synthesis or purchase of fragments) needed to synthesize a decapeptide, for example. As mentioned before (section '8.1.2 Economic analysis of peptide synthesis'), the selling price of a peptide increases radically with an increase in peptide size. This means that the profit margin of coupling two peptide fragments consisting of 20 amino acids each together will be larger than that of coupling two peptide fragments consisting of 5 amino acids each together. Naturally, the higher the profit margin, the more room there is for other

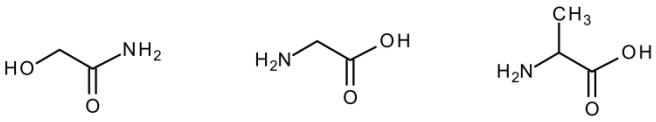
costs. We therefore do believe that it would be interesting, both for a small or large company, to start synthesizing peptides consisting of around 40 amino acids.

8.2.7 Possible solutions for current bottlenecks

In this study we chose to prevent product precipitation in order to minimize practical complications. Also the substrates were fully soluble at the concentrations used. This, however, leads to the use of large amounts of THF in order to attain the yearly production quantity of peptides. This solubility problem will only increase as the size of the produced peptide increases. A possible solution for this bottleneck is the use of a different solvent: a solvent that will better solubilize peptides such as dimethylformamide or dichloromethane. There is a big risk, however, that these solvents denature the enzyme. Another possibility would be to carry out the enzymatic coupling reaction in a solid-to-solid manner, in which the reaction mixture consists of solid substrates and products either including a solvent in which the substrates are only slightly soluble or with no added solvent at all (*i.e.* by using a liquid substrate) (Gill & Vulfson 1993; López-Fandiño *et al.* 1994; Halling *et al.* 1995; Eichhorn *et al.* 1997; Erbeldinger *et al.* 1998; Michielsen *et al.* 2000; Erbeldinger *et al.* 2001; Ulijn *et al.* 2002). This would, however, need some further investigation on, for example, the rate of substrate dissolution and product crystallization, and the effect of possible substrate and / or product precipitation on the catalyst, with respect to the desired coupling reaction.

A possible bottleneck is the relatively low coupling rate of Z-Phe-OCam and Phe-NH_2, as the reaction is inhibited by both products. One way to increase the peptide synthesis rate is to selectively remove either one or both the products during the reaction. Especially the selective removal of glycolamide would be advantageous as this was found to be a stronger inhibitor than the dipeptide (Vossenberg *et al.* submitted-b). The structure of glycolamide is, however, very similar to small amino acids like glycine and alanine (Figure 8.10). If these amino acids are used as one of the substrates, a selective adsorbent for glycolamide would not be an option. Nevertheless, when coupling peptide fragments together, the substrates will differ greatly from glycolamide. Another option would be to

selectively remove the desired peptide product during the reaction. This would minimize product inhibition as well as facilitate DSP.



glycolamide glycine alanine

Figure 8.10. Structures of glycolamide, glycine, and alanine**.**

8.3 Concluding remarks

One could of course argue for different choices in the process design, such as decreasing the 99 % conversion target as it has a major effect on the enzyme concentration or reaction time needed. In addition, we could have chosen to deviate from equimolarity of the starting concentrations of the reactants, again in order to save in enzyme requirement or reaction time. Nevertheless, in this process design we especially focused on keeping the DSP stream as clean as possible in order to minimize the DSP costs.

The choices that lead to the proposed process design in this work as well as the estimates given for the reactor size, volumes of solvent, amounts of substrate, enzyme and molecular sieves, were based on a specific coupling reaction. Nevertheless, we do believe that this case study gives a good impression of the various choices that have to made when designing a process for enzymatic peptide synthesis and the implications of these choices.

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Summary

Peptides, in particular oligopeptides, play an important role in the fields of health care, nutrition and cosmetics. Chemical synthesis is currently the most mature technique for the synthesis of peptides that range in length from 5 to 80 amino acids. Chemical synthesis is, however, expected to be more and more combined with enzyme-catalyzed synthesis, resulting in chemo-enzymatic approaches towards peptide synthesis. The racemization that hampers chemical synthesis can be prevented by forming the peptide bond enzymatically.

In the work in this thesis the bioprocess engineering aspects of a specific chemo-enzymatic peptide synthesis route are studied. In this route, an N-protected, C-terminally activated amino acid is coupled with a C-protected amino acid nucleophile. The coupling step is catalyzed by Alcalase. The initial idea was to also enzymatically catalyze the activation of the amino acid, which is used in the coupling step, and to carry out the coupling and activation steps in one pot. In the work of Chapter 2 lipase B from *Candida antarctica* (CalB) and Alcalase were used as a model system for such a one-pot chemo-enzymatic peptide synthesis, in order to investigate the (in)compatibility between the two enzymes. The rate of activity loss of native and immobilized CalB in the absence and presence of native and immobilized Alcalase was calculated from the rate of triacetin hydrolysis. It was shown that native Alcalase degrades native CalB under aqueous conditions. Immobilization of both or either CalB or Alcalase onto macroporous beads, however, effectively prevented hydrolysis of CalB by Alcalase.

Due to the current impracticality of the enzyme-catalyzed activation step, the rest of the work in the thesis focuses on the Alcalase-catalyzed coupling step. The non-aqueous coupling in tetrahydrofuran (THF) of phenylalanine amide (Phe-NH_2) and the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam) was used as a model coupling reaction.

In protease-catalyzed peptide synthesis the availability of water is essential, as a compromise must be made between on the one hand the overall enzymatic activity and, on the other hand, the rate of product synthesis. Water is essential for enzyme activity, but at the same time causes hydrolytic side reactions. In the work of Chapter 3 the model coupling reaction was catalyzed by cross-linked enzyme

aggregates of Alcalase optimized for use in organic media (Alcalase CLEA-OM) at a range of water activity (a_w) values, including the coupling in the presence of molecular sieves (*i.e.* at very low a_w values). The rate of peptide synthesis could not be increased by increasing a_w values without significantly increasing the rate of hydrolysis, *i.e.* without significantly decreasing the synthesis / hydrolysis (S/H) ratio. Hydrolysis (in the present system, only the activated substrate, not the dipeptide product, may be hydrolyzed) was found to dominate above $a_w \approx 0.2$. To prevent hydrolysis, the presence of molecular sieves was found to be necessary. Nevertheless, the use of molecular sieves over longer periods of time should be carefully considered as they may dehydrate and thereby inactivate the enzyme in time.

In the work of Chapter 4, besides CLEA-OM, also other Alcalase formulations were used to catalyze the model coupling reaction. The reaction was done in the presence of molecular sieves (*i.e.* under near-dry conditions). Hydration prior to drying (with anhydrous *tert*-butanol and anhydrous THF) of the Alcalase formulations resulted in a significant increase in rate of the subsequent dipeptide synthesis. Without such initial hydration, the enzymes seem to lack the water needed to maintain their catalytically active conformation. Repeated use in the presence of molecular sieves, without intermediate rehydration, led to inactivation of the enzyme. For three enzyme formulations this inactivation could be counteracted by intermediate rehydration. Inactivation of another enzyme formulation, Alcalase immobilized onto dicalite, was only partially reversible by hydration. Alcalase immobilized onto dicalite was found to be initially the most active in dipeptide synthesis. Nevertheless, due to its small particle size and its lack of operational stability, this formulation may not be the best choice for the synthesis of dipeptides in neat organic media on a large scale. The most promising enzyme formulation for this is Alcalase covalently immobilized onto macroporous acrylic beads (in this thesis abbreviated as Cov) due to its reasonable activity, its seemingly good operational stability, and its practical size and uniform spherical shape.

If, for economic reasons, Cov should be reused repeatedly for dipeptide synthesis in organic media, its operational stability is important and thus its activity should

not decrease significantly. The long-term stability and reuse of hydrated Cov in THF was investigated in the work of Chapter 5. Cov was incubated with and without molecular sieves (beads or powder) in anhydrous THF. After different incubation periods in THF, the enzyme activity was determined in an aqueous environment. In addition, Cov was repeatedly recycled in order to examine its reusability. The effect of reuse on the aqueous activity of Cov and on the Cov-catalyzed model coupling reaction in near-anhydrous THF was studied. Without molecular sieve beads, Cov hardly inactivated in THF. Nevertheless, when Cov was incubated with molecular sieve beads in THF in rotating reaction vials, Cov lost activity over time. Mechanical damage of Cov by the molecular sieve beads was found to be the main reason for the instability of Cov. In order to reuse Cov for the model coupling reaction in the presence of molecular sieves, it needs to be rehydrated in between the batches. Nevertheless, each intermediate rehydration step also caused a small but significant enzyme activity loss.

In the work of Chapter 6, the coupling kinetics of the model coupling reaction, catalyzed by hydrated Cov, were investigated. Near-anhydrous conditions were maintained by a carefully chosen amount of molecular sieve powder (in contrast to molecular sieve beads, molecular sieve powder does not lead to mechanical damage of Cov). Kinetic characteristics were determined from reaction time courses up to full conversion at various initial concentrations of substrate and product. These progress curve data were fitted with different kinetic models to determine which of these models best approximates the kinetic properties of the immobilized Alcalase with respect to the coupling under study. It was found that the kinetics of the coupling can be described well with a two-substrate kinetic model with two inhibitory products. To reduce the effect of the product inhibition on Cov, a reactor should be designed in which at least glycolamide is selectively removed, as it was found to be the strongest inhibitor.

In Chapter 4 it was shown that molecular sieves dehydrate and thereby reversibly inactivate the enzyme. In the work of Chapter 7 the effect of enzyme dehydration by molecular sieves on the Cov-catalyzed model coupling reaction was studied in detail. The dehydration kinetics of Cov by different amounts of molecular sieve powder were determined by incubating Cov with molecular sieve powder for

different periods of time. Subsequently, the remaining coupling activity of Cov was measured. Dehydration-induced inactivation of Cov by molecular sieve powder seemed to occur in three phases: (1) an initial, rapid, major dehydration-induced inactivation that takes place during the first activity measurement (1 h), (2) a phase of first-order inactivation (20 h), and (3) a relatively low plateau phase in activity. These dehydration kinetics were incorporated into the reaction kinetics model described in Chapter 6. The resulting model was then used to fit progress curve data of the model coupling reaction in the presence of different amounts of molecular sieve powder. Using the estimated parameter values, the model was used to predict independent data sets and found to work well.

The work of Chapter 8 is a case study about a process design for enzymatic peptide synthesis, which is based on the findings of the previous chapters. The choices with regard to Alcalase formulation, type of reactor, way to control the water content, and whether or not to recycle the enzyme, are discussed. In addition, an estimate is given for the reactor size, volumes of solvent, amounts of substrate, enzyme and molecular sieves, needed in order to produce a specific demand for peptides. We believe that this case study gives a good impression of the various choices that have to be made when designing a process for enzymatic peptide synthesis and the implications of these choices.

Samenvatting

Peptiden, in het bijzonder oligopeptiden, zijn belangrijk voor verschillende toepassingsgebieden: gezondheidszorg, voeding en cosmetica. Chemische synthese is momenteel de meest gangbare techniek voor de synthese van peptiden die in lengte variëren van 5 tot 80 aminozuren. Er wordt echter verwacht dat chemische synthese steeds vaker gecombineerd zal worden met enzym-gekatalyseerde synthese, resulterend in chemo-enzymatische peptidesynthese. Racemisatie, een ongewenst bijverschijnsel bij chemische synthese, kan voorkomen worden door de peptidebinding enzymatisch te vormen.

In het werk beschreven in dit proefschrift worden de bioproces-technologische aspecten van een bepaalde chemo-enzymatische peptidesynthese bestudeerd. In deze synthese, wordt een N-beschermd, C-terminaal-geactiveerd aminozuur gekoppeld aan een C-beschermd nucleofiel aminozuur. Deze koppeling wordt gekatalyseerd door Alcalase. Het oorspronkelijke idee was om ook de activering van het te koppelen aminozuur enzymatisch te katalyseren, en om de koppelings- en activeringsstap op elkaar af te stemmen en in één pot uit te voeren.

In het werk beschreven in hoofdstuk 2 worden lipase B uit *Candida antarctica* (CalB) en Alcalase gebruikt als modelsysteem voor een dergelijke chemo-enzymatische één-pot-synthese. Aan de hand van dit modelsysteem werd de (in)compatibiliteit tussen beide enzymen onderzocht. De mate van activiteitsverlies van natieve en geïmmobiliseerde CalB in de af- en aanwezigheid van natieve en geïmmobiliseerde Alcalase werd berekend uit de snelheid van triacetine-hydrolyse. Er werd aangetoond dat natieve Alcalase, natieve CalB degradeert. Immobilisatie van zowel CalB als ook Alcalase op macro-poreuze bollen, of immobilisatie van één van beide, kan de hydrolyse van CalB door Alcalase voorkomen.

Aangezien het op dit moment niet praktisch haalbaar is om de activeringstap enzymatisch te katalyseren, richt de rest van het proefschrift zich op de Alcalase-gekatalyseerde koppelingsstap. De koppeling van fenylalanine-amide (Phe-NH_2) met de carbamoylmethyl-ester van fenylalanine (waarvan de aminogroep beschermd is met benzyloxycarbonyl, Z-Phe-OCam) in droge tetrahydrofuran (THF) werd gebruikt als een modelreactie voor de koppeling.

In protease-gekatalyseerde peptidesynthese is de beschikbaarheid van water essentieel, omdat er een compromis gesloten moet worden aangaande algemene enzymactiviteit enerzijds en het onderdrukken van ongewenste nevenreacties anderzijds. Water is essentieel voor de enzymactiviteit maar tegelijkertijd veroorzaakt het hydrolyse van het geactiveerde substraat. In het werk beschreven in hoofdstuk 3 wordt de model-koppelingsreactie gekatalyseerd door gecrosslinkte Alcalaseaggregaten welke geoptimaliseerd zijn voor gebruik in organische oplosmiddelen (Alcalase CLEA-OM). Deze Alcalase-aggregaten werden gebruikt bij verschillende wateractiviteiten (a_w). Zeer lage a_w-waarden werden gerealiseerd door het gebruik van moleculaire zeven. De peptidesynthese kon niet worden versneld door het verhogen van de a_w-waarden zonder tegelijkertijd de snelheid van substraathydrolyse te verhogen. Dat houdt in dat hogere a_w-waarden de verhouding tussen synthese en hydrolyse (de S/H-ratio) ongunstig beïnvloeden. Hydrolyse bleek boven $a_w \approx 0.2$ te domineren (in het huidige systeem kan alleen het geactiveerde substraat, en niet de dipeptide, worden gehydrolyseerd). Om hydrolyse te voorkomen bleek de aanwezigheid van moleculaire zeven noodzakelijk. Niettemin moet het gebruik van moleculaire zeven over langere tijd zorgvuldig overwogen worden aangezien ze het enzym in de tijd uitdrogen en daarmee het enzym inactiveren.

In het werk beschreven in hoofdstuk 4 worden, naast Alcalase CLEA-OM, ook andere Alcalase-formuleringen gebruikt om de model-koppelingsreactie te katalyseren. De reactie werd uitgevoerd in aanwezigheid van moleculaire zeven en dus onder 'droge' omstandigheden. Voorafgaand aan het drogen met watervrij *tert*-butanol en THF werden de Alcalase-formuleringen gehydrateerd; deze hydratatie resulteerde in een significante snelheidstoename van de dipeptidesynthese. Zonder een dergelijke hydratatie lijkt er onvoldoende water beschikbaar te zijn om een katalytisch-actieve configuratie van de enzymen mogelijk te maken. Hergebruik van een enzym in aanwezigheid van moleculaire zeven en zonder tussentijdse rehydratatie bleek te leiden tot inactivatie van het enzym. Voor drie enzymformuleringen kon deze inactivatie worden tegengegaan met tussentijdse rehydratatie. Inactivatie van een andere enzymformulering, Alcalase geïmmobiliseerd op dicaliet, was slechts gedeeltelijk omkeerbaar door

tussentijdse rehydratatie. Alcalase geïmmobiliseerd op dicaliet bleek initieel het meest actief in dipeptidesynthese. Door de geringe deeltjesgrootte en het gebrek aan operationele stabiliteit is deze formulering echter niet de beste keuze voor de synthese van dipeptiden in organische oplosmiddelen op grote schaal. De meest belovende enzymformulering hiervoor is covalent geïmmobiliseerde Alcalase op macro-poreuze bollen (in dit proefschrift afgekort als Cov); deze formulering onderscheidt zich door zijn redelijke activiteit, goede operationele stabiliteit, en praktische grootte en uniforme bolvorm.

Indien om economische redenen Cov herhaaldelijk hergebruikt moet worden voor dipeptide synthese in organische oplosmiddelen, dan is operationele stabiliteit van belang en dus moet de activiteit niet aanzienlijk verminderen. De stabiliteit op de lange termijn en het hergebruik van gehydrateerde Cov in THF wordt in het werk beschreven in hoofdstuk 5 onderzocht. Cov werd geïncubeerd met en zonder moleculaire zeven (bollen of poeder) in watervrij THF. Na verschillende incubateperioden in THF werd de enzymactiviteit in een waterige omgeving bepaald. Bovendien werd Cov herhaaldelijk hergebruikt om de herbruikbaarheid te onderzoeken. Het effect van hergebruik op de waterige activiteit van Cov en op de Cov-gekatalyseerde koppelingsreactie in vrijwel watervrij THF werd bestudeerd. Zonder moleculaire zeef-bollen inactiveerde Cov nauwelijks in THF. Wanneer Cov echter werd geïncubeerd met moleculaire zeef-bollen in THF in roterende reactievaatjes, verloor het enzym activiteit in de tijd. Mechanische beschadiging van Cov door de moleculaire zeef-bollen bleek de belangrijkste reden voor deze instabiliteit te zijn. Om Cov te hergebruiken voor de model-koppelingsreactie in aanwezigheid van moleculaire zeven, moet de enzymformulering tussentijds gerehydrateerd worden. Toch leid elke tussentijdse rehydratatie stap ook tot een klein maar significant verlies van enzymactiviteit.

In het werk beschreven in hoofdstuk 6 wordt de koppelingskinetiek onderzocht van de door Cov-gekatalyseerde modelreactie. Vrijwel watervrij THF werd bereid door een zorgvuldig gekozen hoeveelheid moleculaire zeef-poeder toe te voegen (in tegenstelling tot moleculaire zeef-bollen leid moleculaire zeef-poeder niet tot mechanische beschadiging van Cov). Kinetische eigenschappen werden bepaald door de reactie tot volledige substraatconversie te volgen bij verschillende

beginconcentraties van substraat en product. Verschillende kinetische modellen werden gebruikt om de experimentele data te beschrijven; op basis hiervan werd het model geselecteerd dat de kinetische eigenschappen van Cov, met betrekking tot de koppeling onder studie, het beste benaderde. De koppelingskinetiek bleek goed beschreven te kunnen worden door een kinetisch model met twee substraatafhankelijkheden en met twee productremmingstermen. Met dit model kon de experimentele data goed beschreven worden. Om het effect van productremming op Cov te verminderen dient een reactor ontworpen te worden waarin met name glycolamide selectief wordt verwijderd, aangezien dit de sterkste remmer bleek te zijn.

In hoofdstuk 4 werd aangetoond dat moleculaire zeven het enzym uitdrogen en daarmee reversibel inactiveren. In het werk beschreven in hoofdstuk 7 wordt het effect van enzymuitdroging door moleculaire zeven op de Cov-gekatalyseerde model-koppelingsreactie bestudeerd. De kinetiek van de dehydratatie van Cov door moleculaire zeef-poeder werd bepaald door Cov met verschillende hoeveelheden moleculaire zeef-poeder gedurende verschillende perioden te incuberen. Vervolgens werd de resterende koppelingsactiviteit van Cov gemeten. Door uitdroging veroorzaakte inactivatie van Cov, door moleculaire zeef-poeder, bleek in drie fasen voor te komen: (1) een eerste, snelle, forse uitdroging en inactivatie die bijna instantaan plaatsvindt (1 u), (2) een fase van eerste-orde-inactivatie (20 u), en (3) een relatief lage plateaufase in activiteit. Deze dehydratiekinetiek werd geïntegreerd in het eerdere reactiekinetiekmodel zoals dat beschreven werd in hoofdstuk 6. Het gecombineerde model werd vervolgens gebruikt om de voortgang van de modelreactie, in aanwezigheid van verschillende hoeveelheden moleculaire zeef-poeder, te beschrijven. Na de vaststelling van parameterwaarden, werd het model tenslotte gebruikt om onafhankelijke data te voorspellen. Het model bleek daarbij goed te werken.

Het werk dat beschreven wordt in hoofdstuk 8 is casus over een procesontwerp voor enzymatische peptidesynthese die gebaseerd is op de bevindingen in voorgaande hoofstukken. De keuzes met betrekking tot Alcalaseformulering, type reactor, controle van het watergehalte, en eventueel hergebruik van het enzym, worden besproken. Bovendien wordt een schatting gegeven voor de

reactorgrootte, hoeveelheden oplosmiddel, substraat, enzym en moleculaire zeven, die nodig zijn om een specifieke hoeveelheid van peptiden te produceren. Wij zijn van mening dat deze casus een goede indruk geeft van de verschillende keuzes die gemaakt moeten worden bij het ontwerpen van een proces voor enzymatische peptide synthese en de implicaties van deze keuzes.

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Petra

Curriculum Vitae
Publications
Training Activities

Curriculum Vitae

Petra Vossenberg was born on February 16, 1985 in Terneuzen, The Netherlands. At the age of ten, she moved to Leipzig, Germany, where she attended the Leipzig International School. She received her International Baccalaureate Diploma in 2003. In the same year she left Leipzig and started studying Biotechnology at Wageningen University, specializing in process engineering. Her B.Sc. thesis, entitled "enzymatic synthesis in microreactors", was carried out at Food and Bioprocess Engineering, Wageningen University. Petra obtained her B.Sc. Biotechnology degree, *cum laude*, in June 2007. Her M.Sc. thesis, entitled "spatial modeling of stem cell growth in scaffold systems for tissue engineering", was carried out at both the Systems and Control of Wageningen University and the Department of Tissue Regeneration, Institute for Biomedical Technology, of the University of Twente. During her internship, Petra worked on the integration of reaction and separation for peptide production, at DSM Food Specialties in Delft. In August 2008, she received her M.Sc. Biotechnology degree, *cum laude*. Directly hereafter, she started her Ph.D. at Bioprocess Engineering, investigating the bioprocess engineering aspects of chemo-enzymatic peptide synthesis. The results can be found in this thesis. On the 1st of October 2012 Petra started working as researcher at FrieslandCampina.

Publications

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. Process design for enzymatic peptide synthesis in near-anhydrous organic media. Submitted.

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. Effect of enzyme dehydration on Alcalase-catalyzed dipeptide synthesis in near-anhydrous organic media. Submitted.

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. Kinetics of Alcalase-catalyzed dipeptide synthesis in near-anhydrous organic media. Submitted.

Vossenberg P, Beeftink HH, Nuijens T, Quaedflieg PJLM, Cohen Stuart MA, Tramper J. Dipeptide synthesis in near-anhydrous organic media: Long-term stability and reusability of immobilized Alcalase. Submitted.

Vossenberg P, Beeftink HH, Nuijens T, Quaedflieg PJLM, Cohen Stuart MA, Tramper J. 2012. Performance of Alcalase formulations in near dry organic media: Effect of enzyme hydration on dipeptide synthesis. Journal of Molecular Catalysis B: Enzymatic 78:24-31.

Vossenberg P, Beeftink HH, Nuijens T, Cohen Stuart MA, Tramper J. 2012. Selecting optimal conditions for Alcalase CLEA-OM for synthesis of dipeptides in organic media. Journal of Molecular Catalysis B: Enzymatic 75:43-49

Vossenberg P, Beeftink HH, Cohen Stuart MA, Tramper J. 2011. Immobilization to prevent enzyme incompatibility with proteases. Biocatalysis and Biotransformation 29(6):288-298.

Vossenberg P, Higuera GA, van Straten G, van Blitterswijk CA, van Boxtel AJB. 2009. Darcian permeability constant as indicator for shear stresses in regular scaffold systems for tissue engineering. Biomechanics and Modelling in Mechanobiology 8:499–507.

Swarts JW, **Vossenberg P,** Meerman MH, Janssen AEM, Boom RM. 2008. Comparison of two-phase lipase-catalyzed esterification on micro and bench scale. Biotechnology and Bioengineering 99(4):855-861.

Overview of completed training activities

Discipline specific activities	
Thermodynamics in biochemical engineering	2008
A unified approach to mass transfer	2008
IBOS conferences	2008 - 2012
Advanced course biocatalysis	2009
Netherlands Biotechnology Congress	2010
Summer school biotransformations	2011
General courses	
Ph.D. competence assessment	2008
Teaching and supervising thesis students	2009
VLAG Ph.D. week	2009
Project- and time management	2009
Effective behavior in your professional surroundings	2009
Techniques for writing and presenting a scientific paper	2010
Career assessment	2011
Career perspectives	2012
Optional	
Research methods biomolecules and interfaces	2008
Bioprocess Engineering brainstorm days	2009 - 2012
Bioprocess Engineering Ph.D. study tour to the USA	2010
Teaching	
Introduction to process engineering	2008 - 2010
Bioprocess design	2009

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