Immobilisation of Metallo-enzymes and their Application in Non-natural Conversions

Martin Bakker
"Immobilisation of metallo-enzymes and their application in non-natural conversions"

van Martin Bakker


4. In veel gevallen van enzymen die oplosbaar zijn gemaakt in organische oplosmiddelen betreft het waarschijnlijk een colloïdale oplossing.


8. Het valt niet te verwonderen dat fenylaceetamide in aanwezigheid van nitrilase of nitrilhydratase niet wordt gedehydrateerd tot fenylacetonitril, aangezien deze reactie thermodynamisch uiterst ongunstig is.


9. Studenten willen in de toekomst graag tot de YUPpen, DINKies of LIMEn behoren, ook al zeggen ze soms van niet.

10. Sinterklaas is echt en de kerstman niet.
Immobilisation of metallo-enzymes and their application in non-natural conversions

Proefschrift

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List of abbreviations

ACN  acetonitrile
A. melleus  *Aspergillus melleus*
A. oryzae  *Aspergillus oryzae*
AOT  aerosol OT: sodium bis(2-ethylhexyl)sulfosuccinate
Brij 52  diethylene glycol monocetyl ether
CaAc  calcium acetate
ChirocLEC PC  CLECs of *Pseudomonas cepia* lipase
CLCs  “home made” crosslinked enzyme crystals
CLEAs  crosslinked enzyme aggregates
CLEC  commercially prepared crosslinked enzyme crystals
CPA  carboxypeptidase A
CPO  chloroperoxidase from *Caldariomyces fumago*
cU  one unit will liberated one μmol tyrosine per minute
DEAE  diethylaminoethyl
DMSO  dimethylsulfoxide
EDTA  ethylenediaminetetraacetic acid
ee  enantiomeric excess
EGDME  ethylene glycol dimethyl ether
E_{immob}  immobilisation efficiency: U_{found in foam}/U_{bound in foam}
EP100  macroporous polypropylene
<table>
<thead>
<tr>
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<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>FAGLA</td>
<td>3-(2-furylacryloyl)-glycyl-L-leucine amide</td>
</tr>
<tr>
<td>fU</td>
<td>one unit will hydrolyse one µmol FAGLA per minute</td>
</tr>
<tr>
<td>Hypol</td>
<td>a water-activated derivative of toluene-2,6-diisocyanate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>MCD</td>
<td>monochlorodimedone</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Pepti-CLEC™-TR</td>
<td>CLECS of thermolysin</td>
</tr>
<tr>
<td>Prot</td>
<td>protein</td>
</tr>
<tr>
<td>PUR</td>
<td>polyurethane</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>STY</td>
<td>space-time yield</td>
</tr>
<tr>
<td>TBHP</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>t-BuOH</td>
<td>tert-butyl alcohol</td>
</tr>
<tr>
<td>t-BuOMe</td>
<td>tert-butyl methyl ether</td>
</tr>
<tr>
<td>TOF</td>
<td>turnover frequency</td>
</tr>
<tr>
<td>TON</td>
<td>turnover number</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>OP</td>
<td>1,10-phenanthroline</td>
</tr>
<tr>
<td>U</td>
<td>µmol substrate converted per min</td>
</tr>
<tr>
<td>UV-Visible</td>
<td>Ultra Violet Visible</td>
</tr>
<tr>
<td>V-phytase</td>
<td>Vanadium substituted phytase</td>
</tr>
<tr>
<td>Y_immob</td>
<td>fraction of protein that was bound to the foam</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

General introduction

Demand for efficient and practical methods for the synthesis of enantiomerically pure L-
aminos acids is still growing. The major field of application of amino acids is in the food and feed
industry whereby the L-amino acids are used for pharmaceutical purposes such as infusions an
therapy. Historically, various methods have been used for the production of natural amino
acids: extraction from protein hydrolysate, fermentation, chemical synthesis or the use of
enzymatic methods. For the latter, many proteases are readily available, that in their natural
function mediate the degradation of proteins to enantiopure amino acids. Besides proteases
other enzymes, e.g. acylases, amidases, hydantoinases and transaminases, have been used to
produce enantiopure amino acids via hydrolysis.

Many industrially relevant hydrolases such as proteases, lipases and aminoacylases, are
derived from fungal or bacterial sources, which are eminently suitable for rapid large-scale
culture. The enzymes for e.g. food, laundry and agrochemical purposes are produced in bulk
quantities and generally used without any purification. Enzymes for the production of
pharmaceuticals are generally produced in relatively small quantities. In this case a high
degree of purity is necessary which in most cases gives higher production costs which also
significantly influences the cost-price of the final product. Ultimately, however, the commercial
viability of bioconversions depends on the enzyme costs per kg of product which are determined
by the cost of the enzyme and its productivity.

The productivity of an enzyme catalysed reaction is, in turn, determined by enzyme
productivity (g product per g biocatalyst per unit time) which is related to the turnover number
(TON) or better, the turnover frequency (TOF), and the volumetric productivity or space-time yield
(STY). The turnover number (TON: moles of product produced per mole of catalyst) and turnover
frequency (TOF: moles of product per mole of catalyst per unit time) are important parameters for
determining the catalyst cost for a process. Immobilisation techniques have resulted in
biocatalysts with increased operational stability compared with the native enzymes. This
significantly reduces costs due to a higher turn-over number, although the price of the carrier
should also be taken into account. The second important parameter, STY, is the amount of
product produced in a certain volume per unit of time (which has a bearing on the required installation costs). The industrial biocatalytic process will stand or fall depending on the above mentioned parameters and still further improvement is necessary to screen for new processes and/or increase viability of the existing processes.

**Introduction to metallo-hydrolases**

Hydrolases account for 75% of the worldwide sales of industrial enzymes. Proteases represent 60% of this group and amylases, lipases and other hydrolytic enzymes are responsible for the other 40%. Proteases are widely applied in the food, detergent and leather industries. They are available from a wide diversity of sources: plants, animals and microorganisms.

According to the nomenclature committee of the International Union of Biochemistry the hydrolases are classified as group 3 and proteases fall into subgroup 4 and 5 of the hydrolases. Due to their huge diversity as regards structure and function they should rather be classified on 1) type of reaction catalysed, 2) chemical nature of the catalytic site and 3) evolutionary relationship with reference to structure; instead of only the classification based on the reaction catalysed. For example, proteases can be roughly subdivided into two major groups, endopeptidases and exopeptidases, depending on their site of action (see Figure 1.1). The endopeptidases act at the internal peptide bonds in the polypeptide chain and the presence of free amino or carboxyl groups has a negative effect on enzyme activity. The exopeptidases act only close to the termini of the polypeptide chains, based on their site of action at the N or C terminus for aminopeptidase and carboxypeptidase, respectively. Both endo- and exopeptidases can be further subdivided into four subgroups based on their catalytic mechanism: serine-, cysteine-, aspartic- and metalloproteases.

![Regioselectivity of hydrolysis for endo- and exopeptidases](image)

Figure 1.1: Regioselectivity of hydrolysis for endo- and exopeptidases

The serine proteases chymotrypsin, subtilisin, carboxypeptidase C (and Y) and penicillin amidases possess a catalytic serine in the active centre. The cysteine proteases, such as papain, calpain, clostripain and streptopain, contain cysteine in the active centre involved in the catalytic process. The aspartic proteases (mainly endopeptidases), e.g. pepsin, retropepsin, thermopsin, *Aspergillus* proteinase A and rennin, usually depend on two aspartic groups for their catalytic
Introduction

activity. The active site of the metalloproteases, such as aminopeptidase, carboxypeptidase A and thermolysin, contain a metal ion (commonly zinc). The metalloprotease thermolysin (E.C. 3.4.24.4) is one of the subjects of this thesis.

Thermolysin

Thermolysin (Bacillus thermoproteolyticus neutral proteinase; E.C. 3.4.24.4) is an extracellular enzyme, which has been isolated from the culture medium of the thermophilic microorganism Bacillus thermoproteolyticus Rokko.\textsuperscript{21} The enzyme is a thermostable metalloprotease containing a zinc ion in the active site, which is essential for its hydrolytic activity.\textsuperscript{21,22} Thermolysin has a broad specificity and cleaves amide linkages involving most amino acid residues with the exception of lysine, arginine and cysteine. The enzyme preferentially cleaves peptide bonds in which the nitrogen is contributed by an amino acid with a hydrophobic side chain, such as leucine of phenylalanine.\textsuperscript{21,23} It does not cleave peptides with either free amino or free carboxylic groups and has a strict requirement for the L-configuration. Because of its specificity the enzyme is more useful for the secondary cleavage of smaller peptides derived from protein hydrolysis than as a tool for the hydrolysis of long peptide chains.\textsuperscript{24}

The application of thermolysin in biotransformations is much wider than cleavage of peptides. It is able to catalyse peptide synthesis and is used on an industrial scale in the synthesis of aspartame, a low-calorie sweetener.\textsuperscript{25,26}

Aminoacylase

The aminoacylases (N-acyl-L-amino acid amidohydrolase; E.C. 3.5.1.14) contain, analogous to thermolysin, zinc in the active site. The enzymes are classified as hydrolases and are either D- or L-specific. Aminoacylases resemble aminopeptidases in their mode of action as shown in the Figure 1.1 for exopeptidases, although acylated amino groups are preferred in the active site.\textsuperscript{27}

Aminoacylases are applied in industrial biotransformations, e.g. in the production of L-amino acids by the enantioselective hydrolysis of N-acetyl-D,L-amino acids.\textsuperscript{28,29} L-specific aminoacylases are found in nature in mammalian sources (pork kidney and bovine liver) and microorganisms (Aspergillus, Pseudomonas and Comamonas species\textsuperscript{30-32}). For large scale applications, microbial acylases are invariably used because of their better availability and lower price compared with the mammalian sources.\textsuperscript{28,33} Two L-aminoacylases are readily available from Aspergillus strains: Aspergillus melleus and Aspergillus oryzae, respectively.\textsuperscript{7} These enzymes are very similar but subtle differences become apparent in practical application.

D-aminoacylases are mainly produced by Pseudomonas, Streptomyces, Alcaligenes and Stenotrophomonas species. These enzymes can be used in principle for the analogous production of D-amino acids, e.g. D-valine, D-phenylglycine and D-p-hydroxyphenylglycine, which are used
Chapter 1

as pharmaceutical and agrochemical intermediates. However, there are no commercially available D-aminoacylases as yet.\textsuperscript{34}

Enzymes in organic solvents

Enzymes are traditionally used in aqueous medium.\textsuperscript{7,10} History shows us that the use of enzymes in biotechnological processes focused on their hydrolytic activity towards biopolymers in aqueous conditions.\textsuperscript{3-10} Changing the reaction medium into organic solvents to perform the reverse reaction widened the potential of biocatalysis enormously.\textsuperscript{7} Ester synthesis in the presence of pig pancreas lipases was demonstrated nearly a century ago.\textsuperscript{35} However, the application of biocatalysts in organic synthesis was hampered by the low solubility of organic compounds in aqueous media. In 1930 Sym presented a possible solution by using a monophasic water/acetone medium for his enzyme catalysed reaction.\textsuperscript{36} In 1966 Dastoli et al. introduced the use of chymotrypsin and xanthine oxidase as catalysts suspended in organic solvent\textsuperscript{37}, but this work was not responded either. The seminal work by Klibanov, who presented a large number of applications of biocatalysts in non-aqueous media, greatly stimulated further research in the field of biotransformations.\textsuperscript{38} Nowadays, the ability of enzymes to act as specific and enantioselective catalysts in aqueous, as well as in organic media is widely recognised.\textsuperscript{16} Hence, slightly more enzyme catalysed reactions in organic solvents were reported in the last decade than biotransformations in water.\textsuperscript{7,11,39-42}

Aminoacylase and thermolysin are both used as catalysts in organic solvents. Aminoacylase from pork kidney has been used for the enantioselective production of N-acetyl-L-methionine in organic medium containing 3 % of water.\textsuperscript{43-45} Thermolysin was used in the synthesis of Z-Asp-Phe-OMe (an aspartame precursor) from Z-Asp and L-Phe-OMe in a water saturated ethyl acetate solution\textsuperscript{46}, although under aqueous conditions this process is much more favourable. In aqueous buffer the product forms an insoluble salt with the D-phenylalanine methyl ester coproduct which drives the equilibrium towards the peptide synthesis.\textsuperscript{47}

The use of biocatalysts in organic synthesis has many benefits, e.g. a high degree of enantio- and regioselectivity,\textsuperscript{13,48} reduction of the total number of steps and mild conditions of pH and temperature which are important when labile reactants/products are involved.\textsuperscript{50} However, there are some disadvantages associated with biotransformation in organic solvents. Besides different energetics of substrate desolvation\textsuperscript{51} and mass-transfer effects between substrate and enzymes, enzyme denaturation in many hydrophilic and hydrophobic solvents is a major problem. In addition, conformational changes of the enzyme in water-immiscible organic solvents might occur causing a loss of selectivity.\textsuperscript{52-54} Moreover, enzymes generally exhibit much lower activities in organic solvents than in water.\textsuperscript{46} To increase enzyme stabilities and activities in organic solvents immobilisation, colyophilisation of enzymes with salts or surfactants or enzyme modification can be applied.\textsuperscript{55,56}
Introduction

Immobolisation of enzymes

In industrial processes economics generally dictate low costs leading to recycling of the (expensive) biocatalyst. One way to facilitate recycling is via immobilisation.\textsuperscript{56,57} Chibata introduced the common definition for immobilised enzymes: enzymes physically confined or localised in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously.\textsuperscript{58} Moreover, immobilisation of enzymes may improve their activity and stability in organic solvents.\textsuperscript{56} However, it has also some disadvantages which are summarised, together with the advantages, in Table 1.1.

Table 1.1: Advantages and disadvantages of enzyme immobilisation

<table>
<thead>
<tr>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Enzymes are reusable</td>
<td>* Additional costs of the method and carrier</td>
</tr>
<tr>
<td>* Processes can be operated continuously and</td>
<td>* Increased catalyst volume</td>
</tr>
<tr>
<td>readily controlled</td>
<td>* The insoluble catalyst may be diffusion limited for substrates</td>
</tr>
<tr>
<td>* Easy separation of products</td>
<td>* Loss of enzyme activity (in some cases even selectivity)</td>
</tr>
<tr>
<td>* Effluent problems and materials handling</td>
<td>* A time consuming effort to obtain a suitable carrier</td>
</tr>
<tr>
<td>are minimised</td>
<td></td>
</tr>
<tr>
<td>* Increased enzyme stability (in some cases</td>
<td></td>
</tr>
<tr>
<td>also activity)</td>
<td></td>
</tr>
</tbody>
</table>

The decision to use either immobilised or free enzyme in a given procedure depends upon economical, technical and practical considerations.\textsuperscript{10} The more expensive the enzyme preparation, the greater the incentive to use it in immobilised form to minimise the catalyst costs per unit weight of product. However, the disadvantages, such as the loss of enzyme selectivity and activity, could disfavour immobilisation.\textsuperscript{59} Furthermore, certain enzymes can be produced cheaply in bulk quantities and immobilisation is not necessary in this case.\textsuperscript{8}

Immobolisation often improves the stability of the enzyme, or at least apparently does. For example, fixation of proteases onto supports prevents the autolysis which occurs readily in solution.\textsuperscript{60} There is no universal method to immobilise enzymes. A wide range of methodologies is available and each has its advantages and disadvantages. These should be considered for each enzyme to be immobilised and for the process in which the catalyst will be used. Several immobilisation methods are summarised in Figure 1.2 and will be discussed next.

Adsorption

Adsorption of enzymes is based on an interaction of biocatalyst and support. The bonding is generally weak and affected by pH, temperature, salts and solvents. Carriers that have been used range from inorganic oxides to organic polymers. For example, Accurel EP100\textsuperscript{61}
(polypropylene), silica, mesoporous molecular sieves, Eudragit S-100 (an enteric methacrylate polymer) and Teflon have been used. Most are macroporous and adsorb the enzyme at the pore surface. However, immobilisates prepared via adsorption can be subject to leaching of the enzyme in aqueous medium. Adsorption followed by crosslinking prevents leaching and can increase enzyme stability although the enzyme activity can be adversely affected.

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Figure 1.2: Summary of enzyme immobilisation methods

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Introduction

Ionic bonding

Enzymes are polyions and will bind to oppositely charged supports. Ionic bonding of enzymes is (operationally) much more stable than physical adsorption, although the stability of the preparation may be sensitive to pH and ionic strength. Hence, the carrier is easy to regenerate. The carrier can have anionic or cationic character; the enzyme loading and activity depends on the charge of the enzyme. Examples of anion exchange supports are DEAE-cellulose\textsuperscript{67}, chitosan beads\textsuperscript{68}, amberlite (IRA)\textsuperscript{69} and DEAE-sephadex.\textsuperscript{70} Typical cation exchange supports are cellulose phosphate\textsuperscript{71}, amberlite (ICR)\textsuperscript{72} and dextran sulfate.\textsuperscript{73}

Covalent attachment

The immobilisation of enzymes via covalent attachment is probably the most widely applied method.\textsuperscript{10} The bond is created via a reaction of reactive groups at the protein surface, e.g. a N-terminus, lysine NH\textsubscript{2} groups or hydroxyl, sulphhydryl or phenolic functional groups, with water insoluble matrices. Examples of carriers which are used for the covalent immobilisation of enzymes are: activated cellulose\textsuperscript{74}, dextran\textsuperscript{75} and starch\textsuperscript{76} (via formation of reactive imidocarbonates from hydroxyl groups and cyanogen bromide), isocyanate prepolymer\textsuperscript{s}\textsuperscript{77} (Hypol 3000) and the very popular Eupergit C (copolymer of methylacrylamide containing oxirane groups) (see Figure 1.3).\textsuperscript{78}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{Covalent attachment of enzymes on Eupergit C}
\end{figure}

Eupergit C is a copolymer of methylacrylamide, N,N'-methylene-bis(methacrylamide) and monomers containing oxirane groups. The protein NH\textsubscript{2} groups react with the oxirane groups via nucleophilic substitution. Sulphhydryl and hydroxyl groups of enzymes also react with the epoxide groups but their reactivity is very low compared with that of the amino groups.

The advantages of covalent attachment of enzymes as immobilisation method are increased stability, caused by the strong interaction with the carrier. However, the strong bond decreases the enzyme’s flexibility, which may have an effect on the activity. Moreover, the multipoint attachment may result in structural changes resulting in loss of activity. Finally the, often expensive, carrier material cannot be reused.
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Crosslinking

Crosslinking involves the attachment of molecules of enzyme to each other via covalent bonds. Bi- and multifunctional compounds are used as reagents for the intermolecular crosslinking of enzymes, such as, for example, glutaraldehyde, diazobenzidine, tannic acid and dimethyl adipimidate. They usually react with amino groups on the outer surface of the protein. The method is very attractive due to its simplicity and almost pure immobilised enzyme is obtained. The crosslinking of dissolved enzymes is hard to control, however. Crosslinking of protein precipitates affords a degree of control over the properties of the final product. Recently, good results were obtained with so-called crosslinked enzyme aggregates (CLEAS).

Robust catalysts have been obtained via the crosslinking of enzyme crystals (CLECS). A serious obstacle to the application of this method is that most enzymes may form a number of crystal shapes. For example, thermolysin may crystallise in five different crystal forms depending on the conditions used (Figure 1.4).

Figure 1.4: Different crystal shapes of thermolysin; (a) needle (b) rice shape (c) hexagonal rod (d) tetragonal bipyramid (e) shag or bow shape

The size, shape and thickness of the crystals can be influenced by varying the pH, temperature, enzyme concentration and the addition of precipitating agents, buffers and adjuvants. Although CLECS are chemically and mechanically quite robust catalysts, the development of suitable protocols for crystallisation and crosslinking is lengthy and labour-intensive, which is a distinct disadvantage compared with CLEAS.

Entrapment

Entrapment is a mild immobilisation method that does not involve any modification or binding of the enzyme. The method is based on the encapsulation of enzymes into matrixes,
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membranes or lattice structures to prevent enzyme leakage from the porous structure. Entrapment in a polymeric matrix can be accomplished by polymerising, e.g. thermally or chemically, a monomer solution containing the enzyme. Many variations are possible with regard to the constraining structure, e.g. gel\textsuperscript{,}\textsuperscript{86} or fiber entrapment\textsuperscript{87} and microencapsulation (see next section). Compared with the other immobilisation methods this procedure can be applied to every kind of enzyme, which makes the method more or less universal. However, a disadvantage of this method is that a large pore size could cause enzyme leakage whereas a small pore size could inhibit the diffusion of large substrate molecules into the matrix. Furthermore, the conditions during polymerisation can destroy enzymes.

Microencapsulation

Semipermeable polymer membranes, prepared from nylon\textsuperscript{88}, cellulose nitrate\textsuperscript{89} or 1,6-diaminohexane and adipoyl chloride\textsuperscript{90}, have been used to enclose enzymes in microcapsules (entrapment b in Figure 1.2). The usual pore size ranges from 1 to 100 nm, which is sufficient to prevent enzyme leakage and allow substrates to dialyse freely across the membrane.\textsuperscript{90} Microcapsules can be compared with artificial cells. The use of permeated, whole dead cells is a closely related technique. A small pore size can be a disadvantage for high molecular weight substrates. Furthermore, this entrapment method similar to fiber entrapment is limited to aqueous media.

Perspective

There are many methods available to immobilise enzymes. Which one is to be preferred to immobilise a certain enzyme, depends on its specific application. Essentially it involves a trial and error approach. The advantages of enzyme immobilisation for industrial application are enhanced stability and reusability, resulting in cost savings. These potential advantages could be (partially) offset by a decrease in catalytic performance. Effects that influence the enzyme activity can be classified as conformational and steric, partitioning, microenvironmental and mass-transfer effects.\textsuperscript{91,92} The selectivity and catalytic activity of the immobilised enzyme preparations should be measured and compared with the native enzymes. Due to the changes in conformation, character and behaviour, immobilised enzymes should be treated as new catalysts. The choice of a suitable support material to immobilise on, the water content and the selection of the organic solvent are crucial for the use of immobilised enzymes in organic media.\textsuperscript{51,56}

For the application of immobilised biocatalysts a very important parameter is the volume of the prepared biocatalyst. The productivity per gram enzyme versus their immobilised form should be compared. If the weight of the carrier contains \textgreater 99\% of the biocatalyst, the enzyme activity could be high but the activity per gram of biocatalyst (productivity) and the STY will be too low (most of the reactor volume is taken by the catalyst). This is one of the main advantages of using
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the crosslinking technique, e.g. CLEAs and CLECs, were the biocatalyst is almost 100% pure enzyme.

**Colyophilisation of enzymes with adjuvants**

Besides immobilisation, colyophilisation with a suitable adjuvant is a fast and easy technique to increase enzyme stability in organic solvents.\(^{93,94}\) Upon lyophilisation the water is removed from an enzyme containing buffer salts and surfactants whereby a coated enzyme is formed. Colyophilisation of enzymes has similarities with immobilisation; however, we reasoned that colyophilisation was initially invented to stabilise and solubilise enzymes in organic solvents (and extract water), whereas immobilisation is focused on stability and reusability.\(^{95-97}\) The enzyme surfactant conjugates, prepared via colyophilisation of enzymes and surfactants, are compatible with anhydrous organic solvents\(^{98-100}\), whereby the conjugation can involve anionic, non-ionic and covalent bonding. Many variations are possible to create a protective layer around the enzyme molecules and increase stability. A wide variety of additives or lyoprotecting agents, e.g. carbohydrates\(^{101}\), cyclodextrins\(^{102}\), polymers\(^{103}\), salts\(^{104}\) and crown ethers\(^{105-107}\) have been colyophilised with enzymes to this end.

Besides protecting the enzyme in organic solvent, the colyophilisation method stabilises the enzyme structure during drying. For instance, Khmelnitsky and coworkers\(^{104}\) colyophilised enzymes with large amounts of salt, which resulted in a drastically enhanced rate in transesterification. Protection from inactivation by the organic solvent and the stabilisation of the enzyme native structure during lyophilisation was suggested to explain the results.

**Enzyme modification**

Chemical modifications of enzymes can be achieved by either covalent binding of surfactants to amino groups at the protein surface\(^{108,109}\) (comparable with the preparation of enzyme surfactant conjugates) or changing an amino acid residue or a metal ion in the active site of the enzyme.\(^{110}\) Modification as a technique could increase enzyme stability in organic solvents analogous to immobilisation and colyophilisation. Varying the physico-chemical properties of the enzyme via immobilisation or colyophilisation the methods can also be regarded as enzyme modifications. However, we would rather define enzyme modification as altering the enzyme structure and properties fundamentally such that the modified catalyst often does not have much in common with the native enzymes.\(^{108}\) In most cases the method was applied to increase or change enzyme selectivity.\(^{108-110}\) However, the studies on semisynthetic and chemically modified enzymes were mainly directed towards gaining insights into the catalytic mechanism of the enzymes rather than creating new synthetic biocatalysts for preparative use.\(^{111-112}\)

Several methodologies have been developed during the last decade to modify enzymes chemically.\(^{108-111}\) Because suitable enzymes are difficult to obtain, we have undertaken to modify
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the catalytic properties of an available metalloenzyme, thermolysin, by substitution of the catalytic metal atom. The first known example of a successful metal-substitution was published by Kaiser who introduced redox activity into a metallohydrolase by preparing copper (II)-exchanged carboxypeptidase A. Suddenly vanadate-incorporated phytase was introduced to perform redox activity. Hence, we reasoned that it should be possible to modify the natural proteolytic activity of thermolysin into redox activity.

Scope of this thesis

The dual issues of immobilisation of metallo-hydrolases and application of the immobilisate in organic solvents are interwoven throughout this thesis. Our major theme is the use of the readily available industrial catalysts, aminoacylase and thermolysin, in non-natural reactions in anhydrous organic media.

The first enzyme to be studied was the zinc-containing aminoacylase from Aspergillus species. In its natural function this enzyme hydrolyses N-acetyl-l-amino acids. We wished to use aminoacylase as an enantioselective acyl transfer catalyst, a function that is, until now, generally performed by lipases.

To stabilise the catalyst in anhydrous organic media its immobilisation was investigated (Chapter 2 and 3). Aminoacylase proved to be capable of acylating a wide range of arylalkanols efficiently and with high enantioselectivity (Chapter 4).

The colyophilisation of aminoacylase and various surfactants resulted in superactivated conjugates (Chapter 5) that proved to be highly efficient transesterification catalysts with high productivities and high space-time yields.

Thermolysin, a zinc containing endoprotease, was immobilised using various methods (Chapter 6) and the resulting preparations were used as ammonolysis catalysts. In order to obtain redox activity in proteases we tried to substitute the zinc atom of thermolysin with other metals/salts (Chapter 7). The apo-enzyme of native and immobilised enzyme was prepared and the catalytic centre was reconstructed afterwards. The modified thermolysin was tested as a catalyst for oxygen transfer reactions with $\text{H}_2\text{O}_2$.

This thesis contains results of the exploitation, the scope of substrates, the advantages and limitations of two readily available metallo-hydrolases in organic synthesis.

References

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Introduction

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Chapter 2

Immobilisation of glycosylated enzymes into polyurethane foams#

Abstract

Glycosylated enzymes, viz. aminocyclase from *Aspergillus melleus*, chloroperoxidase from *Caldariomyces fumago* and phytase from *Aspergillus ficuum*, were covalently immobilised into polyurethane foams with a high enzyme loading of up to 0.2 g protein per g dry foam. The immobilisation efficiency (retained activity) ranged from 100% at a low loading to 60% at high loadings. In contrast with many other immobilisation methods no leaching of the enzyme from the support took place under reaction conditions. In short, an universal method for the immobilisation of enzymes from fungal sources was developed, affording a highly active, stable and reusable biocatalyst.

# In cooperation with Fred van de Velde
Chapter 2

Introduction

Enzymes are gaining in importance as catalysts in (industrial) organic synthesis. For optimum performance they are often used in immobilised form. A milestone in the use of immobilised enzymes in biotransformations was the Tanabe process for the production of L-amino acids (Figure 2.1) using an immobilised aminoacylase developed by Chibata and coworkers.\(^3\)\(^4\)

\[
\begin{align*}
\text{R-COOH} & \xrightarrow{\text{aminoacylase}} \text{R-COOH} + \text{R-COOH} \\
\text{HN-CH}_3 & \text{NH}_2 & \text{HN-CH}_3 & \text{O} & \text{CH}_3 & \text{COOH} \\
N\text{-acetyl-} & \text{L-amino acid} & N\text{-acetyl-} & \text{D-amino acid} \\
D/L\text{-aminoacid} & + \text{CH}_3\text{COOH} & \\
\end{align*}
\]

Figure 2.1: Production of L-amino acid via an aminoacylase-catalysed resolution of N-acetyl-D/L-amino acids.

In comparison with their native form immobilised enzymes offer several advantages, such as enhanced stability, easier product recovery and purification, the possibility of continuous processes and repetitive enzyme use. However there are also disadvantages, such as the additional costs of the carrier, loss of activity due to immobilisation, a reduction of the activity per unit volume and weight, and the occurrence of diffusion limitations.

The immobilisation of enzymes can be classified into three approaches: support binding methods (covalent bonding, adsorption), crosslinking (e.g. crosslinked enzyme crystals) and entrapment (in gel lattice, microcapsules). The covalent attachment of enzymes into polyurethane foams by an reaction with the isocyanate groups was developed and patented by Wood and coworkers. The method involves mixing of an isocyanate prepolymer with the enzyme solution. The curing reaction of the prepolymer is accompanied by the reaction of isocyanate groups with amine and/or hydroxyl groups of the enzyme, which attach it to the polyurethane matrix (Figure 2.2). Water, which is introduced with the enzyme solution, reacts with the isocyanate groups under the formation of CO\(_2\). Evolution of the latter causes the curing polymer to become porous, forming a spongelike matrix. The physical properties of the polyurethane foams can be influenced by the addition of different amounts of additives, such as initiators, surfactants, and blowing agents.

Covalent attachment in polyurethane foams has been used to immobilise various enzymes, e.g. β-D-galactosidase\(^6\), cellulase\(^7\), amylglucosidase\(^8\), phosphotriesterase\(^9\) and lipases.\(^10\) In all cases reported the enzyme loading was in the order of 1 mg protein per gram foam, which is prohibitive low for many applications. We rationalised that the attachment of glycosylated enzymes to the polymer backbone would be facilitated owing to the large number of reactive hydroxyl groups in the carbohydrate mantle. Moreover, since the attachment of the enzyme to the polymer would be
Immobilisation of glycosylated enzymes into polyurethane foams

primarily by its carbohydrate residues the structural integrity of the protein and, hence, its catalytic activity would not be unduly disturbed.

\[
\begin{align*}
\text{NH}_2-\text{Enzyme} & \xrightarrow{\text{H}_2\text{O}} \text{R}^\text{N} \equiv \text{C} = \text{O} & \text{R}^\text{N} - \text{Enzyme} \\
\text{R}^\text{N} = \text{C} = \text{O} & \xrightarrow{\text{HO-Enzyme}} \text{R} - \text{NH}_2 + \text{CO}_2 \\
\end{align*}
\]

Figure 2.2: Covalent attachment of enzymes into polyurethane foams.

In this chapter we report the polyurethane foam immobilisation of glycosylated enzymes from fungal sources with a very high loading (up to 0.2 g protein per gram dry foam) without leaching of the enzyme from the carrier being observed during the reaction. The method is suitable for the immobilisation of a variety of glycosylated enzymes and leads to highly active, stable and reusable biocatalysts.

Results and discussion

Immobilisation method

We used the technique developed by LeJeune and Russell\textsuperscript{11} for preparing the foam-immobilised enzymes. This involves mixing of an equal volume of enzyme solution with the prepolymer Hypol 3000 together with an emulsifier (Brij 52; diethylene glycol monooctyl ether) to improve the distribution of the enzyme in the polymerisation mixture. Hypol 3000 is a prepolymer, containing unreacted isocyanate moieties, prepared from polyethylene glycol, trimethylolpropane and an excess of toluene-2,6-diisocyanate. Addition of a compound containing reactive OH or NH\textsubscript{2} functionalities, e.g. a protein, leads to further polymerisation and incorporation of the protein in the resulting polyurethane. Cobalt (II) chloride was used as a stabiliser for aminoacylase\textsuperscript{12} and as a promoter for the polymerisation reaction.\textsuperscript{13} Under these conditions foams were formed with an open pore structure and with a narrow pore size distribution.\textsuperscript{9} Variation of the prepolymer, the amount of water or different surfactants can effect the physical properties of the foam, such as porosity, density, and surface area.

Immobilisation of glycosylated enzymes

Many industrially relevant enzymes are derived from fungal sources. Many are extracellular enzymes that are easy to obtain in large amounts from fermentation broths\textsuperscript{14} since their secretion from the cell is facilitated by the glycosyl moieties. Owing to the large number of reactive
carbohydrate hydroxyl functionalities glycosylated enzymes are eminently suited for immobilisation by a reaction with the isocyanate groups in a polyurethane prepolymer. It is known from the literature\(^\text{15}\) that sugar moieties react with isocyanate groups. Furthermore, the hydrophilic carbohydrate groups should mix very well with the hydrophilic prepolymer Hypol 3000 containing less than 5 % weight of free toluene diisocyanate.\(^\text{16,17}\)

To test this hypothesis, we have studied the immobilisation of three representative glycosylated enzymes: aminoacylase (E.C. 3.5.1.14), chloroperoxidase (E.C. 1.11.1.10), and phytase (E.C. 3.1.3.8). These enzymes are known to be heavily glycosylated, having a sugar content of, respectively, 18% for chloroperoxidase from \textit{C. fumago}\(^\text{18}\), 25% for phytase from \textit{A. ficuum}\(^\text{19}\), and 46% for aminoacylase from \textit{Aspergillus melleus}.\(^\text{20}\) The hydrolysis of \textit{N}-acetyl-L-methionine was used as an assay for aminoacylase (Figure 2.1; \(R = \text{-CH}_2\text{-CH}_2\text{-S-CH}_3\)) because this allowed us to compare our results with the commercial Tanabe process. Chloroperoxidase, which is known to be an efficient catalyst for a wide variety of synthetically useful (enantioselective) oxygen transfer reactions\(^\text{21,22}\), and vanadate substituted phytase\(^\text{23}\) were studied using the sulfoxidation of thioanisole as a test reaction (see Figure 2.3).

![Sulfoxidation of thioanisole catalysed by either CPO or phytase.](image)

Figure 2.3: Sulfoxidation of thioanisole catalysed by either CPO or phytase.

The amount of protein that could be attached in the polymer was initially investigated, because this could possibly place an upper limit on the activity of the immobilised enzyme. The amount of enzyme that had been fixed to the polymer was determined indirectly, by subtracting the amount of protein found in the wash solvent from the amount of protein added to the polymerisation mixture.

Aminoacylase was captured very efficiently; even at a loading of 190 mg/g no residual protein could be detected in the wash solvent as shown by the straight line in Figure 2.4. Chloroperoxidase was immobilised to a maximum of 25 mg/g; at this loading 82% of the available protein was fixed to the carrier (Table 2.1). The fraction of phytase that became attached to the carrier was rather less and it decreased slightly to 46% when the amount of protein in the polymerisation mixture was increased to 200 mg/g (see Figure 2.4). Typical activities obtained at the highest loading were 86, 7,
Immobilisation of glycosylated enzymes into polyurethane foams

and 8 U/g dry foam for aminoacylase, phytase, and CPO, respectively (unit: μmol substrate converted in 1 min; substrates as explained in the experimental part).

![Graph showing fraction protein in foam (mg/g dry foam) vs fraction protein before immobilization (mg/g prepolymer).]

Figure 2.4. Amount of protein attached to the polyurethane foam during the immobilisation of aminoacylase (○) and phytase (○).

The enzyme loading into the polyurethane foam reflects the carbohydrate content of the enzyme and decreased in the order aminoacylase > phytase > CPO. We also observed that the fraction of CPO and phytase that is bound decreased when the loading was increased, indicating that saturation of the available isocyanate takes place. This could be caused by the considerable amounts of adjuvants (stabilisers e.g. salts, other proteins, carbohydrates and surfactants) and impurities that are present in CPO and, in particular in phytase. We note that stable immobilisation of the crude aminoacylase preparation, which contains large amounts of adjuvant, could not be achieved (data not shown). When partly purified phytase was used instead of crude enzyme powder the average yield increased from 60% to 85%.

Besides the protein loading and the immobilisation yield (Y_immobi; the fraction of protein added to the polymer that becomes bound to the foam) one more parameter is used to describe the immobilisation process: the immobilisation efficiency (E_immobi or retained activity) represents the residual activity of the bound enzyme (E_immobi = U_{found in foam}/U_{bound into foam}).

The immobilisation yields and efficiencies obtained with the three glycosylated enzymes in this present study are summarised in Table 2.1. This table also gives an overview of the results of other foam immobilised enzymes reported in literature (see the introduction for details).
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### Table 2.1: Immobilisation of different enzymes into polyurethane foams

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Ref.</th>
<th>Enzyme loading(^a) (mg/g foam)</th>
<th>Yield(^b) (%)</th>
<th>Efficiency(^c) (%)</th>
<th>Intra/extra Cellular Glycosylation (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoacylase</td>
<td>8</td>
<td>187</td>
<td>100</td>
<td>98</td>
<td>Extra</td>
<td>46</td>
</tr>
<tr>
<td><em>Aspergillus melleus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>2</td>
<td>24</td>
<td>100</td>
<td>100</td>
<td>Extra</td>
<td>18</td>
</tr>
<tr>
<td><em>Caldariomyces fumago</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytase</td>
<td>23</td>
<td>92</td>
<td>60</td>
<td>128</td>
<td>Extra</td>
<td>25</td>
</tr>
<tr>
<td><em>Aspergillus ficuum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>24</td>
<td>0.05</td>
<td>30</td>
<td>83(^d)</td>
<td>Extra</td>
<td>19</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>6</td>
<td>0.4</td>
<td>45</td>
<td>63</td>
<td>Extra</td>
<td>8</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose isomerase</td>
<td>8</td>
<td>(72 U)</td>
<td>47</td>
<td>118</td>
<td>Intra</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptomyces rubiginosus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>25</td>
<td>2.6(^e)</td>
<td>73</td>
<td>100</td>
<td>Extra</td>
<td>-</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
<td>10</td>
<td>1.1</td>
<td>0.11</td>
<td></td>
<td>Extra</td>
<td>-</td>
</tr>
<tr>
<td><em>Humicola lanuginosa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphotriesterase</td>
<td>9</td>
<td>0.38</td>
<td></td>
<td></td>
<td>Intra</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*a:* units reported in literature were when possible converted into mg using the activity of commercially available enzyme samples.

*b:* yield: fraction of protein that was bound to the foam

*c:* efficiency: (U/mg bound enzyme)/(U/mg free enzyme)

*d:* immobilisation efficiency does not change with increasing enzyme loading

*e:* this 2.6 mg commercial preparation contained only 0.04 mg protein

*f:* no information about glycosylation was published
Immobilisation of glycosylated enzymes into polyurethane foams

Table 2.1 clearly shows that the glycosylated enzymes were efficiently immobilised into polyurethane foams. At low loadings (< 10 mg/g) aminoacylase, CPO, and phytase showed an immobilisation efficiency of 100%. All other enzymes given in the table were less efficiently immobilised. Two more extracellular glycosylated enzymes were used in literature studies, viz. amyloglucosidase (19%) and β-D-galactosidase (8%). The low immobilisation yield and enzyme loading obtained for these enzymes could result from the fact that both enzymes are glycoside hydrolysing enzymes which are able to cleave the sugar chains of their neighbours that bind them to the polymeric backbone. Furthermore, the purity of the enzymes was not defined.

In the case of phytase, an immobilisation efficiency of over 100% was obtained suggesting that either purification by selective immobilisation of the phytase occurred or that the immobilised phytase had an enhanced activity compared to the native form. An efficiency above 100% was also reported for glucose isomerase.\textsuperscript{24} For the three glycosylated enzymes used in this study the immobilisation efficiency was monitored as a function of the protein loading (Figure 2.5).

![Figure 2.5](image)

**Figure 2.5** Immobilisation efficiency for the PUR-foam immobilisation of aminoacylase (○), chloroperoxidase (△) and phytase (●) as a function of the enzyme loading.

As shown in Figure 2.5, the immobilisation efficiency decreased with increasing protein loading. One possible reason for this could be stacking of the enzymes on the carrier, which decreases the accessibility of the enzymes close to the carrier. The sugar tails of glycosylated enzymes serve as a spacer and, therefore, reduce the stacking effects. Aminoacylase, which has the highest degree of glycosylation (46%), showed the smallest influence of the enzyme loading on the immobilisation efficiency. A lower degree of glycosylation for phytase (25%) and CPO (18%) resulted in a lower efficiency at a high protein loading. The catalytic efficiency of CPO declined quite severely when the loading was increased.
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One possible reason for the general decline in efficiency with increasing loading could be stacking of the enzymes on the carrier, which could decrease the accessibility of the enzymes close to the carrier. The effect seems to correlate with the carbohydrate content of the enzymes, which is consistent with the notion that the sugar chains serve as a spacer and, therefore, reduce the stacking effects. Aminoacylase, which has the highest degree of glycosylation (46%), showed the smallest influence of the enzyme loading on the immobilisation efficiency. The carbohydrate contents of phytase (25%) and CPO (18%) are approximately half that value and their efficiency at high protein loading is accordingly depressed. We note that the low efficiency reported for β-galactosidase from \textit{Aspergillus oryzae}\textsuperscript{6} at a low loading (0.73 mg/g) is in agreement with its low carbohydrate content.\textsuperscript{26}

The conspicuously fast decrease in efficiency of immobilised CPO (Figure 2.5) may also be caused by its low molecular weight, which may result in an increased stacking of enzyme molecules compared with the other enzymes at the same loading (mg protein/ g foam). It also seems likely that the adjuvants, which are present in commercial CPO interfere with its immobilisation. In contrast with the glycosylated enzymes discussed in this study, entrapment of lipases in polyurethane foam result in their deactivation.\textsuperscript{10} It seems likely that deactivation is caused either by carbamoylation of the active serine by the isocyanate function in the prepolymer or by hindered movement of their lid.\textsuperscript{31}

Recently we demonstrated the scope of our immobilisation method by preparing PUR-foams of the hydroxynitril lyase (E.C. 4.1.2.10) from \textit{Prunus amygdalus} (sweet almond; more than 50% glycosylation).\textsuperscript{32} For the oxynitrilase we observed a 100% yield at a loading of 14 mg protein per g foam and a 90% yield at a loading of 67 mg per g foam, respectively.\textsuperscript{†}

Generally speaking, covalent immobilisation results in a decrease in enzyme activity resulting from transformations of functional groups (e.g. amino groups that react with aldehyde functions from a cross linking reagent or with isocyanate groups) or from structural changes by multiple point attachment. As outlined in Figure 2.2, isocyanate groups can react with both amino and hydroxyl groups of the enzyme during polymerisation, the former being more reactive towards isocyanates. However, glycosylated enzymes are completely covered with a mantle of carbohydrate that hampers access to the NH$_2$ groups of the protein. For example, only 13 amino groups are accessible in CPO\textsuperscript{33}, whereas at least 120 hydroxyl groups are present on its surface.\textsuperscript{18} Hence, we reasoned that glycosylated enzymes are predominantly bound into the PUR-foam via their sugar hydroxyl groups. In short, the sugar coating protects glycosylated enzymes in two ways for deactivation during covalent immobilisation into PUR-foams. First, the sugar hydroxyl groups compete for the isocyanate groups and, therefore, less of the functionally important amino groups in the polypeptide chain are attacked. Second, the sugar coating serves as a spacer between the enzyme and polymeric matrix and prevents or suppresses deactivation resulting from structural changes upon multiple

\textsuperscript{†}The efficiency of the immobilisation method of hydroxynitril lyase in PUR-foams is still under investigation
Immobilisation of glycosylated enzymes into polyurethane foams

point attachment. However, to confirm this hypothesis further experiments need to be performed with deglycosylated enzymes, which will clarify the mechanism of bonding.

Performance of PUR-foam immobilised enzymes

PUR-foam immobilised enzymes are both easy to prepare and handle and, therefore, have considerable potential for industrial applications. This was also noted by Storey and co-workers in connection with the coimmobilisation of cellulase, β-glycosidase and glucose isomerase for the production of high fructose syrup from cellulose. However, the polymerisation reaction is exothermic and, therefore, the large scale immobilisation of heat sensitive enzymes could present problems.

The prepared PUR-foam immobilised enzymes are stable and reusable biocatalysts. As shown in Figure 2.6 no leaching of the enzyme from the polymeric matrix was observed under catalytic conditions, since the oxidation of indole stopped when the PUR-foam immobilised CPO was removed from the reaction mixture.

![Graph showing oxidation of indole catalysed by CPO immobilised into PUR-foam.](image)

Figure 2.6. Oxidation of indole catalysed by CPO immobilised into PUR-foam: wet foam (○); foam dried over P₂O₅ (□); wet foam catalyst removed after 30 min (Δ).

No leaching of the enzyme from the polymeric matrix was observed during one-year storage at 4°C in buffer (no protein was found in the liquid). As Figure 2.6 shows the biocatalyst is still active after drying over P₂O₅ overnight. Furthermore, these dried biocatalysts have an increased stability in organic solvents.

The stability of the PUR-foam aminoacylase was studied in more detail. PUR-foam immobilised aminoacylase was reused 12 times in the hydrolysis of N-acetyl-D/L-methionine (30 minutes reaction time). In order to measure the initial rate, the conversion at that time was around 30%. As shown in Table 2.2 the activity of PUR-foam immobilised aminoacylase did not change
during these 12 reaction cycles demonstrating that it is a perfectly reusable biocatalyst. Moreover, during these experiments we did not observe any mechanical disruption of the catalyst.

Table 2.2: Activity of PUR-foam immobilised aminoacylase in repetitive batch reactions.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg PUR foam</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>94</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>98</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>98</td>
</tr>
<tr>
<td>8</td>
<td>93</td>
</tr>
<tr>
<td>9</td>
<td>98</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>94</td>
</tr>
<tr>
<td>12</td>
<td>96</td>
</tr>
</tbody>
</table>

Manipulating the physical properties of the foam renders the biocatalysts suitable for different purposes and reactor designs. For example crushing the polymers under liquid nitrogen reduced their particle size and resulted in a biocatalyst which settled on the bottom of a reaction vessel (left side of Figure 2.7).

Figure 2.7. Behaviour of PUR-foam bio-catalyst in different forms: crushed foam settles on the bottom (left side); cubes float on top of the reactor (right side).
Immobilisation of glycosylated enzymes into polyurethane foams

Cutting the foam into cubes on the other hand afforded a biocatalyst, which floated on top of the liquid surface (right side of Figure 2.7). Cylindrical foams, prepared by polymerisation in polyethylene tubes, are easy to use in packed bed reactors making them suitable for application in continuous processes. During initial experiments with a 1 meter packed-bed reactor (see Figure 2.8) we did not observe any flow limitations or restrictions resulting from the use of these soft foams.

![Figure 2.8. Packed bed reactor (1 meter) of aminoacylase PUR-foams.](image)

To compare the applicability of our PUR-foam immobilised enzymes with commercially applied immobilised enzymes, we measured the productivity of the PUR-foam immobilised aminoacylase. Its productivity in the conversion of racemic N-acetyl-methionine to L-methionine was 3.4 g (g catalyst)$^{-1}$ d$^{-1}$ which is higher than the 2.15 g (g catalyst)$^{-1}$ d$^{-1}$ for the commercial DEAE-sephadex immobilised aminoacylase.$^3$ Studies on the applications of polyurethane immobilised enzymes in packed bed reactors are under investigation.

Conclusions

Three glycosylated enzymes from fungal sources, viz. aminoacylase from A. melleus, chloroperoxidase from C. fimago, and phytase from A. ficuum, were successfully immobilised by covalent attachment to polyurethane foams. The immobilisation method was very efficient, yielding enzyme preparations with protein contents of up to 0.2 g protein per g dry foam and immobilisation efficiencies (retained activities) ranging from 60% at high protein loadings to 100% at lower loadings. Glycosylation protects the enzyme from deactivation which can result from reaction of
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essential amino functionalities with isocyanate groups or from structural changes arising from multiple point attachment during immobilisation.

The method yields a stable and reusable biocatalyst, which can be used in different reactor designs. The foam immobilised enzymes can be cut into cubes, which float on the surface or can be crushed using liquid nitrogen and dried over P₂O₅ without any loss of activity, yielding a catalyst that forms a suspension in solution. In short, this work shows that these PUR-foam enzymes have considerable potential in industrial biocatalysis.

Experimental procedure

Materials

Aminoacylase from Aspergillus melleus (E.C. 3.5.1.14) and Bradford reagent were purchased from Sigma. The Aspergillus species was dissolved in Tris buffer (pH 7.5; 50 mM containing 1 mM of CoCl₂ for enzyme stabilisation), purified and concentrated using an Amicon Stirred cell, equipped with an ultrafiltration membrane (cut-off 30 kDa). Chloroperoxidase from Caldariomyces fumago (E.C. 1.11.1.10) was isolated and purified as described in the literature. The enzyme solution contained 35.5 mg/ml CPO with Rₜ of 0.95 (Rₜ = purity standard = A₄₀₀/A₂₈₀ = 1.44 for pure enzyme) and an activity of 27 kU/ml according to the standard MCD assay as described by Morris and Hager. Phytase from Aspergillus ficuum (E.C. 3.1.3.8; commercial preparation containing 640 mg protein per g) was a gift from Gist-brocades B.V., The Netherlands, and was used without further purification. Brij 52 (diethylene glycol monocetyl ether) and N-acetyl-L-methionine were purchased from Aldrich. The foamable hydrophilic prepolymer (Hypo™ 3000) was kindly donated by Hampshire Chemical Ltd. Hypol 3000 is a water-activated derivative of toluene-2,6-diisocyanate.

Analysis and equipment

Reversed phase HPLC analysis was performed using a custom-packed Symmetry C₁₈ cartridge (Waters Radial-Pak, 8 x 100 mm, 7 µm) contained in a Waters RCM 8x10 compression unit, with simultaneous detection on a Waters 410 differential refractometer and a Waters 486 tunable absorbance detector with Waters Millennium²² software. The products of N-acetyl-L-methionine hydrolysis were analysed using acetonitrile/phosphate buffer (50 mM; pH 2.2) 7.5:92.5 (v:v) as eluent (flow 1.5 ml/min for the first 4 minutes followed by 3 ml/min), with detection at 210 nm. The products of thioanisole oxidation were analysed using 1,2,3-trimethoxybenzene as internal standard and acetonitrile/water 35:65 (v:v) as eluent (flow 1 ml/min), with detection at 220 nm. UV measurements were performed on a Cary 3 spectrophotometer from Varian. An IKA stirring device, equipped with a double bladed rotor (d=22mm), was used for mixing the enzyme-prepolymer mixtures.
Immobilisation of glycosylated enzymes into polyurethane foams

Enzyme immobilisation

To prepare the foam-immobilised enzymes, the enzyme solution (2.0 ml) was mixed with Brij 52 (50 µl; 4% solution) and CoCl₂ (125 µl; 1 mM solution) in a 50 ml centrifugation tube. Hypol 3000 (2.0 g; preheated to 35 °C to limit handling problems due to its high viscosity) was subsequently added. The mixture was stirred vigorously for 30 seconds to achieve a homogeneous distribution of enzyme within the prepolymer. Polymerisation took place at room temperature. When the increase in volume ceased (1 to 2 minutes) the tubes were stored in ice to minimise enzyme deactivation caused by the increased temperature during the exothermic polymerisation reaction. The foams were stored for at least 2 hours before use. The foam cylinder was cut into round slices of 3 mm thickness (d=25mm), and washed 3 times in 50 ml buffer (50 mM citrate pH 5.0, 50 mM Tris pH 7.0, and 0.1 M formate pH 5.0) for aminoacylase, CPO, and phytase, respectively. Wash solvents were analysed for protein concentration and enzyme activity. The foam slices with immobilised enzyme were stored at 4 °C. For activity analysis a slice was frozen in liquid nitrogen, crushed, and dried overnight over P₂O₅.

Immobilisation of enzymes as a suspension is possible with a high enzyme loading and a good efficiency. In the case that only part of the enzymes is bound to the carrier, the enzyme found in the wash solvent is fully active and easy to recover, concentrate and reuse. The single purification step in the immobilisation of aminoacylase was membrane filtration and is applicable for large scale applications.

Determination of enzyme activity and protein content

The activity of aminoacylase was measured using the standard hydrolysis reaction of N-acetyl-L-methionine as the assay. N-Acetyl-L-methionine (15.7 mM) was dissolved in Tris buffer (5 ml; 50 mM; pH 7.5) and the pH was adjusted with NaOH (1M). Adding HCl (1M; 5ml) after 1 hour quenched the reaction and the conversion was measured by HPLC.

For determining the enzyme stability of recycled aminoacylase a certain amount of immobilised enzyme (20-50 mg) was cut into cubes and used for the hydrolysis reaction. After 30 minutes the cubes were removed from the reaction vessel and reused for the next cycle, whereas the filtrate was analysed by HPLC and leaching of protein was controlled.

For chloroperoxidase the oxidation of thioanisole TBHP in tert-butyl alcohol/water mixtures was used. Citrate buffer (7 ml; 0.1 M; pH 5.0; containing 10 % (v:v) tert-butyl alcohol) and thioanisole (12 mM) were added to an appropriate amount of enzyme preparation. After 15 min stirring at room temperature tert-butyl hydroperoxide (45 mM) was added and the course of the reaction was followed by HPLC.

The activity of immobilised phytase was measured using the earlier discovered peroxidase activity in the presence of vanadate. Formate buffer (7 ml; 0.1 M; pH 5.0; containing 10 µM Na₃VO₄) and thioanisole (5.0 mM) were added to an appropriate amount of enzyme preparation.

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After 15 min stirring at room temperature hydrogen peroxide (5.5 mM) was added and the progress of the reaction was followed by HPLC.

Protein content was determined by the Bradford assay (reliable technique that uses a dye called Coomassie Brilliant Blue G-250, which undergoes a change in colour upon noncovalent binding to protein) using Bovine serum albumin as a standard. Bradford reagent (1.4 ml) was added to an appropriate dilution of enzyme solution (50 μl; protein concentration less than 1 mg/ml). After 30 min incubation at 40 °C the absorbance at 595 nm was measured.

The weight percentage of glycosylated groups for the aminoacylase from A. melleus was calculated from the Bradford assay (0.76 mg protein per g solid) and the amino acid analysis of Gentzen et al.\textsuperscript{20}, referring to 1 mg of protein (by Bradford) corresponds to 0.41 mg of protein.

References

Immobilisation of glycosylated enzymes into polyurethane foams


Chapter 2


Immiscibilisation of aminoacylase

Abstract

Aminoacylase (N-acyl-l-amino acid amidohydrolase; E.C. 3.5.1.14) from Aspergillus melleus was immobilised on DEAE-cellulose, DEAE-Sephadex, Duolite, Accurel EP100, Eupergit C and polyurethane foams in order to increase the stability and operational life of the enzyme in organic solvents. Aminoacylase immobilised on the ionic carrier DEAE-Sephadex had the highest hydrolytic activity and an efficiency (retained activity) of 61 % at a loading of 155 mg protein per g solid. For the covalent attachment of aminoacylase on polyurethane foams a similar efficiency was obtained compared to the ionic carrier but with a higher enzyme loading of 187 mg protein per g of solid.

The catalytic properties of the enzyme changed as a result of immobilisation. The pH optimum of the hydrolysis of N-acetyl-l-methionine shifted from 7.5 for the native enzyme to a more basic or an acidic pH depending on the method of immobilisation. Furthermore, immobilised aminoacylase catalysed enantioselective transesterification more efficiently in moderately hydrophilic organic solvents than in hydrophobic ones, whereas for the native enzyme the reverse was true.
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Introduction

As discussed in Chapter 1 immobilisation of enzymes offers several advantages in biotransformations such as higher enzyme stability and productivity particularly in organic media, facile separations and reuse of the enzyme.\textsuperscript{1-4} Two techniques that are widely used for the immobilisation of enzymes are: (a) ionic bonding to ion exchange resins such as DEAE-Sephadex and Duolite or (b) covalent attachment by reaction of functional groups (e.g. epoxide) on the surface of a polymer. As noted by Tischer and Wedekind\textsuperscript{5}, the choice of immobilisation method strongly depends on the intended application (see Chapter 1).

Aminoacylase (E.C. 3.5.1.14) is used for the industrial production of enantiopure $\text{L}$-amino acids by enzymatic hydrolysis of the corresponding $\text{N}$-acyl amino acids (Figure 3.1).\textsuperscript{1,6} The enzyme from \textit{Aspergillus oryzae} is immobilised on DEAE-Sephadex and used in a fixed-bed reactor for the production of, \textit{inter alia}, $\text{L}$-methionine (Tanabe process).\textsuperscript{1,6,7} This was the first example of the industrial application of immobilised enzymes.\textsuperscript{8} Although DEAE-Sephadex is a relatively expensive support this is offset by the easy regeneration of the deactivated biocatalyst by equilibrating the matrix with fresh enzyme.

\begin{center}
\includegraphics{reaction_diagram}
\end{center}

\textbf{Figure 3.1: Production of $\text{L}$-amino acids with aminoacylase}

Degussa, in contrast, uses the free aminoacylase from \textit{A. oryzae} in an ultrafiltration membrane reactor for the production of certain $\text{L}$-amino acids.\textsuperscript{9,10} The reaction is carried out continuously with economic advantages: efficient utilisation of the enzyme activity, efficient racemization and recycling of undesired acylated D-amino acids (see Figure 3.1).

Based on its availability and unusually wide substrate specificity\textsuperscript{11} we thought that aminoacylase would be a useful catalyst for various biotransformations. In this chapter we describe a comparison of different immobilisation methods, viz., ionic bonding on DEAE-cellulose, DEAE-Sephadex and Duolite, physical adsorption on Accurel EP 100 (polypropene), and covalent attachment to Eupergit C (oxirane acrylic beads) and polyurethane foams, for aminoacylase from \textit{Aspergillus melleus}. The enzyme has roughly the same properties as the
Immobilisation of aminoacylase

protease from the *A. oryzae* species and could be specified as a virtually identical enzyme of the latter biocatalyst.\textsuperscript{12} The aminoacylase from *A. melleus* has recently been used in enantioselective transesterification reactions.\textsuperscript{13} Hence, we tested our immobilised preparations in both the acylation of 1-phenylethanol (1) as well as by the hydrolytic activity (Figure 3.2).

\[
\begin{align*}
\text{OH} & \quad + \quad \text{CH}_2=\text{CH} \quad \xrightarrow{\text{aminoacylase}} \quad \text{O} \\
1 & \quad \text{hexane} \quad \rightarrow \quad 2 & \quad + \quad 3
\end{align*}
\]

Figure 3.2: Transesterification of 1-phenylethanol (1) with vinyl acetate by aminoacylase

Results and discussion

**Immobilisation of aminoacylase**

Aminoacylase from *A. melleus* was attached to the macroporous carriers DEAE-cellulose (copolymer of diethylaminoethyl methacrylate and cellulose), DEAE-Sephadex (crosslinked copolymer of diethylaminoethyl methacrylate and dextran) and Duolite (phenol-formaldehyde resin) by ionic bonding and deposited on Accurel EP100 (macroporous polypropylene) by adsorption. It was also covalently attached to Eupergit C (copolymer of methylacrylamide containing oxirane groups) and polyurethane foams (see Chapter 2). For each carrier we used optimised techniques known from literature.\textsuperscript{7,14-18} In addition, variations were made in the amount of protein (0-198 mg), the salt/ buffer concentration (0.5 M-1.5 M) and the temperature (4-24°C) to obtain the optimum immobilisation method. The native aminoacylase from *Aspergillus melleus* contained 5 mg protein per gram of solid according to the Bradford assay. To prevent low immobilisation yields and efficiencies the enzyme was partly purified by dialysing to remove the adjuvants (stabilisers e.g. other proteins, surfactants and salts).

The activity assay of aminoacylase was usually performed in the presence of added cobalt to increase or maintain enzyme stability.\textsuperscript{6,11} However, we did not add metal ions for the hydrolysis of *N*-acetyl-l-methionine, due to its positive influence on the catalytic activity, which in turn would obscure the effects of immobilisation. The remaining activities after the immobilisation of aminoacylase on different carriers are summarised in Table 3.1. The characteristic features are described, viz., the protein loading, the activity (U/g), the immobilisation yield \((Y_{\text{immoh}}; \text{the fraction of protein added to the carrier that becomes bound to the carrier})\) and the immobilisation efficiency \((E_{\text{immoh}}; \text{or retained activity represented by } U_{\text{found on carrier}}/U_{\text{bound on carrier}})\).
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When comparing the immobilisation of aminoacylase on ionic carriers it becomes obvious that DEAE-Sephadex is the carrier of choice. The highest activity, 44 U/g, was obtained with aminoacylase bonded to Sephadex 50 with an efficiency of 61%. The immobilisation yield, as well as the retained activity decreased with increasing amount of enzyme loading.

The immobilisation on Duolite resulted in a very low retained activity and only a small fraction of the protein was attached to the carrier. The subsequent treatment with glutaraldehyde to crosslink the enzyme to the carrier was omitted because of the low remaining activity.

Table 3.1: The remaining activities of the immobilisation of aminoacylase from A. melleus

<table>
<thead>
<tr>
<th>Batch</th>
<th>Enzyme loading (mg/g solid)</th>
<th>Activity: U/g (μmol sub (g wet min)^{-1})</th>
<th>Y_{immob}^b (%)</th>
<th>E_{immob}^c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-cellulose</td>
<td>25</td>
<td>15</td>
<td>99</td>
<td>65</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>146</td>
<td>21</td>
<td>87</td>
<td>29</td>
</tr>
<tr>
<td>DEAE-Sephadex (A-25)</td>
<td>21</td>
<td>15</td>
<td>99</td>
<td>85</td>
</tr>
<tr>
<td>DEAE-Sephadex (A-25)</td>
<td>148</td>
<td>27</td>
<td>75</td>
<td>38</td>
</tr>
<tr>
<td>DEAE-Sephadex (A-50)</td>
<td>20</td>
<td>23</td>
<td>88</td>
<td>87</td>
</tr>
<tr>
<td>DEAE-Sephadex (A-50)</td>
<td>155</td>
<td>44</td>
<td>71</td>
<td>61</td>
</tr>
<tr>
<td>Duolite A-7</td>
<td>21</td>
<td>0.2</td>
<td>9</td>
<td>0.2</td>
</tr>
<tr>
<td>Duolite S-761</td>
<td>21</td>
<td>0.1</td>
<td>6</td>
<td>0.1</td>
</tr>
<tr>
<td>Accurel EP100</td>
<td>17</td>
<td>1.3</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Eupergit C</td>
<td>8</td>
<td>10</td>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>Eupergit C</td>
<td>15</td>
<td>11</td>
<td>95</td>
<td>20</td>
</tr>
<tr>
<td>Eupergit C (250L)</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Eupergit C (250L)</td>
<td>15</td>
<td>10</td>
<td>51</td>
<td>15</td>
</tr>
<tr>
<td>Polyurethane foam</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Polyurethane foam</td>
<td>187</td>
<td>43</td>
<td>100</td>
<td>60</td>
</tr>
</tbody>
</table>

a: the enzyme loading is specified as the mg of protein attached to the wet carrier. Several batches of purified enzymes were used during immobilisation. The amount of adsorbed water is adjusted for each carrier.

b: yield: the fraction of protein that was bound to the carrier
c: efficiency: (U/mg bound protein)/(U/mg free protein)

Accurel EP100 is a very suitable carrier to adsorb and stabilise lipases, for example, in ammonolysis reactions. However, in our case the immobilisation resulted in an yield of only 10% and an efficiency of 2% resulted. The relatively large pore size of the carrier decreases the specific surface area and therefore, leaded to low yields. Higher yields were obtained with the immobilisation of A. melleus aminoacylase by covalent attachment on Eupergit C (>600 μmol/g
Immobilisation of aminoacylase

dry weight) and the 250L type (>200 μmol/ g dry weight). The protein was covalently bound to Eupergit C by reaction of its free amino groups with the epoxide functionalities of the carrier. Besides covalent binding it is to be expected that additional hydrophobic interactions occur between the hydrophobic backbone of Eupergit (the copolymers of methylacrylamides; see also Chapter 1) and hydrophobic regions of the enzyme. The larger pore size of Eupergit C 250L means that there is a lower surface area available for binding with the enzyme resulting in a lower protein loading and immobilisation yield. Almost all of the aminoacylase was attached to Eupergit C but only an activity of 11 U/g was recovered, which corresponds to an efficiency of 20%, indicating a deactivation of the enzyme upon bonding to the carrier. As with all immobilisation techniques the decrease in activity was presumably due to covalent or ionic binding of essential functional groups of the enzyme with the carrier.2,5,21

The polyurethane foam immobilisation was based on a more hydrophilic carrier (see also Chapter 2), Hypol™ 3000, a partially polymerised material, containing less than 5 % weight of free toluene diisocyanate.23 The highest enzyme loading, 187 mg protein per g dry carrier was obtained by this method with an immobilisation yield of 100%. For the immobilisation of several enzymes on foams (see Chapter 2) we reasoned that one possible explanations for decreased activity at increased enzyme loading was the increased stacking effects of enzyme molecules within the foam. For the carriers DEAE-Sephadex and DEAE-cellulose a similar effect was observed suggesting that the stacking effect was caused a comparable curve (see Figure 2.5) as for the PUR-foam.

We note that the higher enzyme loading and efficiency for the polyurethane foams, DEAE-Sephadex and DEAE-cellulose compared to Duolite and Accurel EP100, suggest that the enzyme prefers hydrophilic carriers above hydrophobic ones.

**Enzymatic properties of immobilised aminoacylase**

The binding of enzymes to a carrier was expected to influence the ionisation state of the charged groups at the enzyme surface and, hence, to shift the pH optimum. We investigated the activity/ pH relationship of aminoacylase immobilised in a number of ways: ionic bonding to the carriers DEAE-Sephadex and DEAE-cellulose, and covalent (multipoint) attachment to Eupergit C and polyurethane foams (Figure 3.3.1).

Compared with the native aminoacylase the pH optimum of all the immobilised preparations became much broader and shifted. When aminoacylase was immobilised on DEAE-cellulose (Figure 3.3.2) the activity was 45 U/g at pH 8, whereas at pH 7.5 an activity of 40 U/g was obtained. The optimal activity versus the pH of the DEAE-Sephadex-aminoacylase moved more to pH 7.0, which was in agreement with published results.7 Similarly for Eupergit C (Figure 3.3.4) a down shift of 0.5 was observed with an activity of 9.4 U/g. For the latter the shift could
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be attributed to the free amino-groups at the surface of the protein reacting with the oxirane
groups of Eupergit.

![Graphs of aminoacylase activities vs pH](image)

Figure 3.3: The pH optima for the hydrolysis of N-acetyl-L-methionine (3-5)

Immobilisation of aminoacylase on PUR-foam (5) shifted the pH optimum in comparison to
the native enzyme from 7.5 to 6.0. The activity was increased by 60% (up to 70 U/g) compared
with the value reported in Table 3.1 (43 U/g measured at pH 7.5).
Immobilisation of aminoacylase

*Application of aminoacylase as a transesterification catalyst*

The immobilised aminoacylase preparations were used in the transesterification of 1-phenylethanol in organic media (see Figure 3.2). The preparations were dried overnight over P₂O₅ to prevent hydrolytic side-reactions. No loss of hydrolytic activity is buffer was observed after drying and rehydration. The aminoacylase on Eupergit C was pre-treated by washing the beads stepwise with small amounts of acetone-water mixtures (up to 2% of water). This method was adopted because it is known that enzymes immobilised on Eupergit lose their catalytic activity when all the water is removed.²⁵

The catalytic performance of the immobilised aminoacylase was compared with that of the native enzyme (see Table 3.2). All preparations acted highly enantioselectively (E>500) in the acylation of 1 with vinyl acetate in hexane or tert-butyl methyl ether. In the presence of the native enzyme, complete conversion (50 %) to the R-enantiomer 2 was achieved within 72 hours, whereas the immobilised preparations gave only partial conversion in 8 days. Aminoacylase immobilised on PUR-foams was completely inactive and no conversion was observed after 11 days.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Solvent</th>
<th>Protein (mg)</th>
<th>Time (d)</th>
<th>Conversion (%)</th>
<th>Time* (d)</th>
<th>Conversion* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td>Hexane</td>
<td>5</td>
<td>3</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DEAE-Sephadex 50</td>
<td>Hexane</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>Hexane</td>
<td>10</td>
<td>8</td>
<td>5</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Eupergit C</td>
<td>Hexane</td>
<td>3</td>
<td>8</td>
<td>17</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>Polyurethane foams</td>
<td>Hexane</td>
<td>38</td>
<td>8</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

* Table 3.2: Aminoacylase-catalysed transesterification of 1-phenylethanol

* a: conversion measured after addition of 20 μl H₂O

Based on the amounts of protein used for each reaction the highest conversions were obtained with aminoacylase immobilised on Eupergit C. The small amounts of water that are known to be adsorbed on Eupergit C presumably stabilise the enzyme. However, the organic medium could strip off the water of the aminoacylase immobilised on Eupergit C and cause deactivation. In comparison with the native enzyme, this could cause the low conversions in the transesterification after 8 days for the immobilised preparations.
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Nearly all the immobilised aminoacylase preparations were activated by the addition of a small amount of water (20 μl). As shown in Table 3.2 the conversion in t-BuOMe increased by more than 25 % upon addition of water. In hexane medium the effect was less pronounced because it does not mix with water. The enhanced enzyme activity upon the addition of water in enzyme catalysed reactions in comparable organic media has been reported before.26-28

The native enzyme is more active in hexane than in t-BuOMe, whereas for the immobilised enzymes the reverse is true. This difference is presumably a consequence of changes in enzyme properties resulting from immobilisation. As shown in Table 3.2, the activity of the enzyme in the transesterification reaction is dependent on the hydrophilic/hydrophobic character of the solvent. The carriers used for immobilisation were in all cases characterised as hydrophilic carriers, which possibly explains why with the immobilised preparations the more hydrophilic solvent (compared to hexane) t-BuOMe was preferred for the transesterifications.

Conclusions

Aminoacylase from Aspergillus melleus was successfully immobilised on DEAE-cellulose, DEAE-Sephadex and PUR-foams with high immobilisation yields and efficiencies for the hydrolysis of N-acetyl-L-methionine. However, immobilisation decreases the catalytic activity and modifies the properties of the enzyme, such as optimum pH.

The immobilised forms of aminoacylase were less catalytically active in transesterifications in organic media than the native enzyme. The immobilised aminoacylase preferred t-BuOMe as solvent to perform the transesterification, whereas for the native enzyme hexane was more suitable.

Experimental procedure

Materials

Aminoacylase from Aspergillus melleus (E.C.3.5.1.14), DEAE-Sephadex and Duolite A7 & S761 were obtained from Fluka. DEAE-Cellulose and N-acetyl-L-methionine were purchased from Aldrich. The Bradford reagent was obtained from Sigma. Cumene and vinyl acetate were purchased from Acros. The foamable hydrophilic prepolymer (HypolTM 3000) was kindly donated by Hampshire Chemical Ltd. Hypol 3000, which is a water derivative of toluene-2,6-diisocyanate. The Eupergit C and Eupergit C 250 l were a gift from Röhm GmbH (Darmstadt, Germany) and Accurel EP100 was donated by Akzo Nobel.

Analysis and equipment

The progress of the reactions was monitored by reversed phase HPLC analysis using a custom-packed Symmetry C18 cartridge (Waters Radial-Pak, 8 x 100 mm, 7 μm) contained in a Waters RCM 8x10 compression unit, with simultaneous detection on a Waters 410 differential
Immobilisation of aminoacylase

refractometer and a Waters 486 tunable absorbance detector at 210 nm with Waters Millenium software. The products of N-acetyl-L-methionine hydrolysis were analysed using acetonitril/phosphate buffer (50 mM; pH 2.2) 7.5:92.5 (v:v) as eluent (flow 1.5 ml/min for the first 4 minutes followed by 3 ml/min).

Samples for monitoring the transesterification reactions were taken from the reaction medium and centrifuged to remove the catalyst. The enantiomeric purity of the alcohols and esters and conversions were analysed by chiral HPLC using a Chiralcel OD column (Daicel Chemical Industries, Ltd., 250 x 4.6 mm), eluent flow 0.6 ml min\(^{-1}\), and detected at 254 nm. A hexane/isopropanol mixture was used as eluent for alcohol 1 and esters.

**Enzyme immobilisation**

The immobilisation of aminoacylase on DEAE-Sephadex was carried out using the method of Chibata.\(^7\) The immobilisation of the enzyme on EP100 was performed according to the method described by Van Rantwijk et al.\(^{16}\) Foam immobilised enzymes were prepared by mixing an equal volume of enzyme solution and prepolymer Hypol\(^{TM}\) 3000 according to the technique earlier described (see Chapter 2).

The immobilisation of aminoacylase on DEAE-cellulose was prepared in a similar way as the enzyme immobilisation on DEAE-Sephadex. DEAE-Cellulose (10 g) was pre-treated with 100 ml NaOH buffer (0.1 M) for 3 hours. After filtration the precipitate was washed with phosphate buffer (0.1 M, pH 7.0) until the solution was neutralised. The resulting solid was shaken with purified aminoacylase (dialysing for a certain time period) for 3 hours at room temperature. After filtration the excess of enzyme was removed by washing with 3 times 200 ml phosphate buffer. From the residue the amount of protein was measured according to the Bradford assay.

Aminoacylase was immobilised on Duolite as described by Olson and Stanley.\(^{31}\) Both phenol-form aldehyde resins were soaked overnight in a 0.1 M NaCl solution and washed afterwards with water. After 4 hours shaking of the aminoacylase solution with the pre-treated resins the solids were filtrated and measured for hydrolytic activity. The crosslinking was performed with a 2 % glutaraldehyde solution during 1 hour.

The covalent attachment of aminoacylases on Eupergit C was performed by shaking a washed native enzyme solution in a phosphate buffer with 1 g of dry polymer. The volume of Eupergit C was 5 ml and of Eupergit C 250L 6 ml. To observe the optimal immobilisation conditions the salt/ buffer concentration was varied from 0.5 to 1.5 M and temperatures of 4 and 24°C were used. Finally, a buffer concentration of 0.5 M (phosphate buffer, pH 7.0) and a temperature of 24°C the highest hydrolytic activity was achieved. After 3 hours the excess of enzyme was filtered off and the solid material was washed with buffer.
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Enzyme activity measurements

The activity of aminocyclase was measured using the hydrolysis of N-acetyl-L-methionine as standard assay. N-acetyl-L-methionine (15.7 mM) was dissolved in TRIS buffer (5 ml; 50 mM; pH 7.5), the pH was adjusted with NaOH (1M) and the enzyme was added to start the reaction. The reaction was quenched after 1 hour by adding HCl (1M; 5 ml) and the conversion was measured by HPLC. The pH optimum was measured varying the pH of the substrate solubilised in TRIS buffer (pH 5 to 9 adjusted with HCl (1M) or NaOH (1M)).

Enzyme catalysed acylation of 1-phenylethanol with vinyl acetate

Aminocyclase immobilised on DEAE-Sephadex or -cellulose, PUR-foams and native enzyme were dried for one night under P₂O₅. The immobilised enzyme preparation on Eupergit C were washed stepwise with 10 ml H₂O/ acetone mixtures, respectively (v:v) 9:2, 6:4, 4:6, 2:8, 0.2:9.8, and dried under vacuum for one hour. A mixture of 0.8 mmol 1-phenylalcohol (1) (100 mg) and 1.5 equivalent vinyl acetate (1.2 mM) were dissolved in 5 ml hexane or t-BuOMe containing 100 mg of native enzyme (5 mg of protein) or 200 mg of dried immobilised catalyst. The course of the enzyme catalysed transesterification reaction was followed by chiral HPLC using cumene as internal standard. After 196 hours a 20 µl of water was added. The enzyme was filtered off after 11 days and the final conversion was measured on chiral HPLC after drying the filtrate with Na₂SO₄. The filtrates were concentrated in vacuum and purified by column chromatography using silica gel and ethyl acetate/petroleum ether 3:1 (v:v) as eluens. The residues resulted in (S)-alcohol ([α]²⁰ = -45°, c =5, MeOH) and (R)-ester ([α]²⁰ = 114°, c = 2, MeOH).

References

Immobilisation of aminocyclase


Chapter 3


25 Personal communication with Röhm GmbH (Darmstadt, Germany). Eupergit C absorbs water in a weight volume of 1 g solid and 3 water. Drying the immobilised enzyme reduces the retained enzyme activity drastically. For data see also Chapter 6.


Chapter 4

Enantioselective aminoacylase-catalysed transesterification of secondary alcohols

Abstract

The aminoacylase (N-acyl-L-amino acid amidohydrolase; E.C. 3.5.1.14) from *Aspergillus melleus*, a readily available inexpensive enzyme, catalyses the transesterification of a wide range of chiral secondary arylalkanols with essentially absolute stereospecificity (E>500). Moreover, the productivity of aminoacylase in the transesterification of most alcohols is substantially higher than these of the lipase catalysed transesterifications.
Chapter 4

Introduction

Enantiomerically pure secondary alcohols are important synthetic intermediates and chiral auxiliaries. They are conveniently accessible by kinetic resolution of the racemate, which has mainly been done by a lipase-catalysed transesterification (see Figure 1). The lipases from Pseudomonas species, porcine pancreas, Candida rugosa and Candida antartica have been used in such reactions and the steric preferences of the most widely used lipases have been characterised. Because secondary alcohols are sluggish reactants and an irreversible reaction is a prerequisite for efficient kinetic resolution, a wide range of activated and/or irreversible acyl donors have been used in combination with lipases, e.g. enol, haloethyl and oxime esters, as well as acid anhydrides. Enol esters, such as vinyl and isopropenyl esters, are the most widely used irreversible acyl donors. They shift the reaction equilibrium towards synthesis because the liberated enol tautomeres to acetaldehyde or acetone, respectively. A disadvantage connected with the use of vinyl esters is that some lipases, notably those from Candida rugosa and Geotrichum candidum, are deactivated due to Schiff’s base formation of lysine residues with acetaldehyde. Acetone, which is the by-product form isopropenyl esters, does not cause deactivation of the enzyme although in most cases the reaction rate was lower due to steric hindrance.

The aminoacylase from Aspergillus melleus (N-acyl-L-amino acid hydrolase, E.C. 3.5.1.14) is a readily available and versatile catalyst for enantioselective hydrolysis of N-acyl amino acids. Although the detailed mechanism for the hydrolysis is not known it is generally assumed that it involves coordination of the carbonyl group of the acylating reagent to the Zn$^{2+}$-ion in the active site, similar to the Zn-dependent proteases. Recent work by the group of Herradon revealed that the aminoacylase is a transesterification catalyst that combines a highly relaxed substrate specificity with a high selectivity for the R-enantiomer of the alcohol (see Figure 4.1).

![Diagram](https://via.placeholder.com/150)

Figure 4.1: Aminoacylase catalysed transesterification of 1-phenylethanol (1) with vinyl ester

As was shown in Chapter 3 aminoacylase immobilisation on different carriers resulted in a considerable decrease in activity in transesterification reactions compared to the native enzyme under similar conditions. Here we present a study of the kinetic resolution of secondary arylalkanols by native aminoacylase mediated transesterification with vinyl acetate.
Enantioselective aminoacylase-catalysed transesterification of secondary alcohols

Results and discussion

*Aminoacylase catalysed transesterifications with different acyl donors*

Initially, we investigated the earlier reported\(^{16}\) aminoacylase-mediated acylation of 1-phenylethanol (1) in toluene more closely. Various solvents were compared with regard to the reaction rate in the acylation of (±) 1 to (R)-1 acetate and (S)-1 by vinyl acetate (Figure 4.1). The highest rates were observed in apolar solvents. Therefore, hexane, in which the reaction took place at an initial rate of 53 μmol (g protein\(^{-1}\)) min\(^{-1}\), was the solvent of choice (Table 4.1 and Figure 4.2). In hexane the reaction was complete within 3 days to convert 50 %, using only one fifth of the amount of catalyst reported by Herradon.\(^{16}\) Figure 4.2 clearly shows that there is an increase of aminoacylase activity with increasing solvent hydrophobicity expressed in terms of log P values.\(^{19,20}\) The low initial rates in acetonitrile, dichloromethane and chlorobenzene were presumably caused by deactivation of the enzyme in more polar organic solvents as mentioned by others.\(^{21}\)

Table 4.1 and Figure 4.2: Aminoacylase catalysed transesterification of 1 with vinyl acetate in different solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial rate (μmol 1 R (g protein(^{-1})) min(^{-1}))</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexane</td>
<td>53</td>
<td>3.5</td>
</tr>
<tr>
<td>isoctane</td>
<td>43</td>
<td>4.5</td>
</tr>
<tr>
<td>toluene</td>
<td>39</td>
<td>2.5</td>
</tr>
<tr>
<td>t-BuOMe</td>
<td>37</td>
<td>2.0</td>
</tr>
<tr>
<td>EGDME</td>
<td>11</td>
<td>0.9</td>
</tr>
<tr>
<td>t-BuOH</td>
<td>11</td>
<td>1.0</td>
</tr>
<tr>
<td>MeCN</td>
<td>4</td>
<td>-0.3</td>
</tr>
<tr>
<td>CH(_2)Cl(_2)</td>
<td>3.9</td>
<td>1.0</td>
</tr>
<tr>
<td>C(_6)H(_5)Cl</td>
<td>11</td>
<td>2.8</td>
</tr>
</tbody>
</table>

(R)-1 were acylated from the racemic and enantiopure substrate at the same rate in separate experiments, whereas the conversion of (S)-1 was too low to measure, which sets the lower limit of the enantiomeric ratio E at 500. This confirms the absolute stereoselectivity of the aminoacylase and also shows that the catalyst is not inhibited by the unreactive (S)-1. The linear progress of the reaction (Figure 4.3) shows that the presence of acetaldehyde has no deleterious
Chapter 4

effect on the biocatalyst. The catalyst could easily be recovered by filtration and the products isolated by column chromatography.

![Graph showing conversion and initial rates over time.](image)

Figure 4.3: Conversions (left) of racemic (-) and (R)-1-phenylethanol (o) and initial rates (right) of the enzymatic reaction to R-acetate 1.

We subsequently studied the effect of the acyl donor on the rate and the enantioselectivity of the acyl transfer. In the presence of a wide range of vinyl esters (Table 4.2) aminoacylase converted racemic 1 into the corresponding (R)-esters and (S)-1 with essentially absolute stereospecificity (E>500).

Table 4.2: Effect of the acyl donor on the aminoacylase mediated acylation of 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate$^a$ (μmol 1 (R) (g protein)$^{-1}$ min$^{-1}$)</th>
<th>Time (days)</th>
<th>Conv. (%)</th>
<th>ee ester (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinyl acetate</td>
<td>53</td>
<td>3</td>
<td>50</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl propionate</td>
<td>67</td>
<td>2</td>
<td>50</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl butyrate</td>
<td>110</td>
<td>1.3</td>
<td>50</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl acrylate</td>
<td>27</td>
<td>7</td>
<td>37</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl crotonate</td>
<td>40</td>
<td>7</td>
<td>45</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl hexanoate</td>
<td>15</td>
<td>4</td>
<td>50</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl octanoate</td>
<td>15</td>
<td>4</td>
<td>50</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl decanoate</td>
<td>14</td>
<td>6</td>
<td>50</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl laurate</td>
<td>35</td>
<td>6</td>
<td>50</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Vinyl benzoate</td>
<td>2</td>
<td>7</td>
<td>14</td>
<td>72</td>
</tr>
<tr>
<td>Isopropenyl acetate</td>
<td>1</td>
<td>7</td>
<td>8</td>
<td>48</td>
</tr>
<tr>
<td>Methyl butyrate</td>
<td>4</td>
<td>7</td>
<td>12</td>
<td>&gt; 99</td>
</tr>
</tbody>
</table>

*a: reaction rate was measured during the first 5 hours*
Enantioselective aminoacylase-catalysed transesterification of secondary alcohols

Vinyl butyrate reacted at twice as fast as the acetate, which has been already reported by Herradon\textsuperscript{16}, but hexanoate and higher esters were much less reactive. Analogous to the lipase-catalysed resolution of 5-phenyl-1-pentan-3-ol\textsuperscript{22}, vinyl acrylate and crotonate reacted slower (70\%) than vinyl acetate. \(\alpha,\beta\)- Unsaturated esters generally react slowly in base catalysed hydrolyses\textsuperscript{23} owing to reduction in the electrophilic character of the carbonyl carbon by delocalisation of the positive charge to the \(\beta\)- carbon atom (Figure 4.4).\textsuperscript{24}

As shown in Figure 4.4 the inductive effect of vinyl acrylate (b) is unfavourable compared with vinyl propionate (c), which explains the decrease in reaction rate. Similarly, the electron withdrawing and donating effects of substituents formed the basis for explaining the rates of hydrolysis of substituted methyl benzoates by general base and nucleophilic catalysis.\textsuperscript{25} Furthermore, the steric hindrance resulting from the comparative inflexibility of the \(\alpha,\beta\)-unsaturated ester could play a role. Similarly, vinyl benzoate gave a low reaction rate and in this case the enantioselectivity was also mediocre. Analogous results were observed in lipase catalysed transesterifications. The reaction rate decreased by a factor 100 when using vinyl benzoate instead of vinyl acetate.\textsuperscript{26}

![Figure 4.4: Resonance contributors of vinyl acrylate (a) and the inductive effect of vinyl acrylate (b) and vinyl propionate (c) in the active site](image)

The effect of the leaving group was briefly investigated. Isopropenyl acetate and methyl butyrate reacted at only a few \% of the rate of vinyl acetate, which renders them unsuitable for this enzymatic resolution.

The scope of aminoacylase-catalysed transesterifications

We have employed vinyl acetate as the standard acylating agent throughout this work in order to compare our results with related work. In order to assess the scope of the aminoacylase-catalysed transesterification, we investigated the acylation of the secondary alcohols 1-12 (see Figure 4.5).

In addition to 1, its homologues 1-phenyl-propanol (2) and \(-\)butanol (3) were likewise converted into the (R)-esters with absolute enantioselectivity. Alcohol 2 was converted at nearly
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the same initial rate as 1, but 3 reacted much slower and complete conversion was not achieved (Table 4.3). We note that the closely related compound 1-phenylbut-3-enol was also converted quite sluggishly in a comparable reaction.\textsuperscript{18} Apparently, a C\textsubscript{3}-group does not fit in the part of the nucleophile subsite that in the 'natural' reaction receives the carboxylate group of N-acetyl-L-methionine.

\begin{align*}
(1) \quad R_1 &= \text{CH}_3 \\
(2) \quad R_1 &= \text{CH}_2\text{CH}_3 \\
(3) \quad R_1 &= \text{CH}_2\text{CH}_2\text{CH}_3 \\
(4) \quad R_2 &= \text{CH}_3 \\
(5) \quad R_2 &= \text{CH}_2\text{CH}_3 \\
(6) \quad R_2 &= \text{OH} \\
(7) \quad R_2 &= \text{OH} \\
(8) \quad n &= 1 \\
(9) \quad n &= 2 \\
(10) \quad n &= 1 \\
(11) \quad n &= 2 \\
(12) \quad n &= 2
\end{align*}

Figure 4.5: Secondary alcohols used for transesterification reactions

When the hydroxyl group was moved to the C-2 position (4 and 5), the acylation became quite tardy and the conversion remained low. Moreover, the enantiomeric ratio decreased by a factor of 50. Surprisingly, 4-phenyl-2-butanol (6), in which the aromatic ring is two carbon atoms away from the alcohol group reacted with absolute enantioselectivity at a rate comparable to 2. The opposite enantiomer, i.e. (S)-6, was preferentially acylated, however. Hence, we conclude that the subsite that in the acylation of 1 receives the methyl group, preferentially binds the phenylbutyl group in the acylation of 6.

Next we investigated the effect of aromatic substitution. A p-isobutyl group (7) had negligible effect on the reaction, but when the phenyl group in 1 was substituted by naphthyl, the results depended on the position of substitution. 1-(2-Naphthyl)-ethanol (8) was completely converted into the (R)-ester but its 1-naphthyl isomer\textsuperscript{18} (9) reacted much slower, although the enantioselectivity remained absolute.

Finally, we attempted the resolution of some bicyclic alcohols. 1-Indanol (10) formed only a minute amount of (R)-product after 7 days, but 1,2,3,4-tetrahydro-1-naphthol (11) gave higher conversions and 1,2,3,4-tetrahydro-2-naphthol (12) was converted at comparable rate to 11. We surmise that the half-chair conformation of the six-membered ring in 11 is more easily accommodated in the active site than the planar five-membered ring in 10. These bicyclic compounds all required the (R)-product.
Enantioselective aminoacylase-catalysed transesterification of secondary alcohols

Table 4.3. Aminoacylase catalysed transesterification of aryl-substituted alcohols

<table>
<thead>
<tr>
<th>Substrate (R,S)</th>
<th>Rate(^a) μmol prod. (g prot.)(^{-1}) min(^{-1})</th>
<th>Time (\text{d})</th>
<th>Conv. (%)</th>
<th>Abs. Config. (\text{b})</th>
<th>Yield(^c) (%)</th>
<th>E(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>53</td>
<td>3</td>
<td>50</td>
<td>R</td>
<td>49</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>7</td>
<td>48</td>
<td>R</td>
<td>44</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>7</td>
<td>22</td>
<td>R</td>
<td>19</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>7</td>
<td>14</td>
<td>(R)(^c)</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>7</td>
<td>16</td>
<td>(R)(^c)</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>7</td>
<td>44</td>
<td>S</td>
<td>43</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>3</td>
<td>50</td>
<td>R</td>
<td>47</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>7</td>
<td>50</td>
<td>R</td>
<td>46</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>7</td>
<td>25</td>
<td>R</td>
<td>22</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>7</td>
<td>5</td>
<td>R</td>
<td>4</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>11</td>
<td>26</td>
<td>7</td>
<td>35</td>
<td>R</td>
<td>30</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>12</td>
<td>19</td>
<td>7</td>
<td>30</td>
<td>R</td>
<td>25</td>
<td>&gt; 500</td>
</tr>
</tbody>
</table>

\(\text{a: reaction rate measured during the first 5 hours}\)
\(\text{b: stereochemical preference}\)
\(\text{c: isolated yield}\)
\(\text{d: calculated according to reference 7}\)

It should be noted that some alcohols, such as 2 and 8, which reacted at an initial rate that is comparable to 1, were slow at achieving a complete conversion. Catalyst deactivation apparently plays only a minor role because the enzyme still retained 40 % of its original hydrolytic activity after seven days in hexane. Inhibition by small amounts of acid, originating from donor hydrolysis by traces of water, could also be involved.

**Comparison of lipases with aminoacylases in transesterifications**

Table 4.4 compares the productivities of aminoacylase-catalysed transesterifications with the corresponding lipase-catalysed reactions.\(^{1,2,5}\) The former were generally higher, albeit sometimes marginally.

The acyl donor can has a markedly effect on the productivity of the enzyme for several alcohols.\(^{5,7}\) For instance, almost no difference was observed in the transesterification of 1 between vinyl butyrate or vinyl octanoate\(^{27}\) with lipases from *Pseudomonas fluorescens* or *cepacia*, whereas lower productivities were obtained with vinyl esters of carboxylic acids with chain lengths larger than 8 carbon atoms.\(^{28}\)
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Table 4.4: Productivities of aminoacylase and lipase of the catalysed transesterification of substrates 1-12.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Productivity aminoacylase ( \frac{g \text{ (g protein)}^{-1} \text{ h}^{-1}}{} )</th>
<th>Productivity(^a) lipase ( \frac{g \text{ (g protein)}^{-1} \text{ h}^{-1}}{} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.207</td>
<td>0.085(^2)</td>
</tr>
<tr>
<td>2</td>
<td>0.083</td>
<td>0.029(^1)</td>
</tr>
<tr>
<td>3</td>
<td>0.037</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>0.074</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>0.154</td>
<td>n.d.</td>
</tr>
<tr>
<td>8</td>
<td>0.082</td>
<td>0.058(^1)</td>
</tr>
<tr>
<td>9</td>
<td>0.026</td>
<td>0.001(^5)</td>
</tr>
<tr>
<td>10</td>
<td>0.009</td>
<td>0.054(^1)</td>
</tr>
<tr>
<td>11</td>
<td>0.061</td>
<td>0.060(^1)</td>
</tr>
<tr>
<td>12</td>
<td>0.051</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\(^{a}\): the productivities of the lipases from Pseudomonas species were only calculated when \(E>500\)

The influence of the structure of the alcohol substrate (1-12) on the enantioselectivity (E) of transesterification for three different lipases is illustrated in Figure 4.7. Most publications gave E values up to 150 whereas the E values >100 are rather inaccurate and even small errors in ee may cause a significant variation in E\(^{29}\) and therefore, we set the maximum at 100. In our case only one enantiomer was detected by aminoacylase and therefore, we used an E value of 500 in previous sections.

Interestingly, none of the enzymes exhibited high enantioselectivity with alcohol 5. The Pseudomonas lipase-catalysed transesterification of substrates 1, 2, 4 and 8-10 resulted in E of 100\(^{1,2,30-32}\) while E values of 73 and 35 were observed with alcohols 7 and 11, respectively.\(^8,33\) The porcine pancreatic lipase gives high enantioselectivities (E=100) with relatively small substrates (1-3) whereas bulky substrates exhibited low enantioselectivity.\(^9\) For example moving from substrate 1 to 6 the E value decreased to 20\(^5,34\) Furthermore, for 8, 10 and 11 at room temperature E values of only 30, 54 and 65, respectively, were observed.\(^35,36\) The Candida antarctica lipase generally showed lower enantioselectivity than the other two lipases.\(^8,10,37,38\) For substrates 1, 4, 8, 9 and 11 an E of 100, 2, 50, 57 and 29 was obtained, respectively.\(^8,39,40\) Aminoacylase from Aspergillus melleus, on the other hand, exhibited broad substrate specificity with absolute stereospecificity for most arylalkanols. It readily accepts a wide range of alcohols containing bulky aromatic groups whereas lipases, are more restricted in scope and/ or exhibit lower E values.\(^9,19,27,36,41\)
Enantioselective aminoacylase-catalysed transesterification of secondary alcohols

Figure 4.7: A 3-dimensional plot of the aromatic substrate selectivity of lipases and aminoacylase in the transesterification reaction of substrates 1-12 (see Figure 4.5)

Conclusion

Aminoacylase from Aspergillus melleus, a readily available enzyme, is a very useful catalyst for enantioselective transesterifications. Without modification the enzyme is stable in organic solvents and acts highly enantioselectively in the acylation of a wide range of secondary arylalkanols. Results are generally superior with regard to both activity and enantioselectivity, compared to the corresponding transformations catalysed by lipases.

Experimental

Materials

Aminoacylase from Aspergillus melleus (E.C. 3.5.1.14) and Bradford reagent were purchased from Sigma. N-acetyl-L-methionine, vinyl propionate, vinyl acrylate, vinyl crotonate, vinyl benzoate, (R,S)-1-phenylethanol, (R,S)-1-phenyl-1-propanol, (R)-1-phenyl-1-butanol, (R,S)-1-(2-naphthyl)ethanol and (R,S)-1,2,3,4-tetrahydro-2-naphthol, were obtained from Aldrich. Cumene, vinyl acetate, vinyl propionate, (R,S)-1-phenyl-2-propanol, (R,S)-1-phenyl-3-butanol and (R,S)-1-(1-naphthyl)ethanol, were purchased form Acros. Vinyl butyrate, methyl butyrate, vinyl laurate, isopropenyl acetate, (R,S)-1-phenyl-2-propanol, (S)-1-phenyl-1-butanol, (R,S)-1-indanol and (R)-1-(1-naphthyl)ethanol were obtained from Fluka and vinyl hexanoate, vinyl octanoate were from Tokyo, Kasei, Kogyo Co. (R,S)-1,2,3,4-tetrahydro-1-naphthol was obtained from Lancaster and 1-(4-isopropylphenyl)ethanol was kindly donated by Hoechst Celanese Corporation.
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Analysis and equipment

Samples for monitoring the transesterification reactions were quenched by removal of the enzyme by centrifugation. The conversion and enantiomeric purity of the alcohols and esters was analysed by chiral HPLC using a Chiracel OD column (Daicel Chemical Industries, Ltd., 250 x 4.6 mm), eluent flow 0.6 ml min⁻¹, and detected on a Waters 486 tunable absorbance detector at 254 nm with Waters Millennium³² software. A hexane/isopropanol mixture (see Table 4.5 and 4.6) was used as eluent for all alcohols and esters.

The progress of the reactions was monitored by reversed phase HPLC using a custom-packed Symmetry C₁₈ cartridge (Waters Radial-pak, 8 x 100 mm, 7 μm) contained in a Waters RCM 8 x 10 compression unit, with detection on a Waters 486 tunable absorbance detector with Waters Millenium³² software. The hydrolysis of N-acetyl-L-methionine was monitored by, reversed phase HPLC on the same Symmetry C₁₈ column, using acetonitrile/phosphate buffer (50 mM; pH 2.2) 7.5: 92.5 (v:v) as eluent (flow 1.5 ml/min for the first 4 minutes followed by 3 ml/min), with detection at 210 nm. Optical rotations were measured using a Perkin Elmer 241 polarimeter.

Aminoacylase activity test

The activity of aminoacylase was measured by the hydrolysis of N-acetyl-L-methionine. N-Acetyl-L-methionine (15.7 mM) was dissolved in Tris buffer (5 ml; 50 mM; pH 7.5), the pH was adjusted with NaOH (1M) to pH 7.5 and enzyme was added. The reaction was quenched after 1 hour by adding HCl (1M; 5 ml) and the substrate concentration was measured by HPLC and the conversion was calculated.

Enzyme catalysed acylation of 1-phenylethanol 1 with enol esters

The experiments were performed at room temperature in a 10 ml reaction vessel containing 50 U aminoacylase from Aspergillus melleus (5 mg protein), 100 mg (0.8 mmol) (±)-1 and 1.2 mmol enol ester dissolved in 5 ml hexane. The course of the reaction was followed by chiral HPLC (Chiracel OD column; Daicel Chemical Industries, Ltd.) using cumene or cymene as the internal standard (see Table 4.5 for the retention time of product and substrates).

After 7 days the reaction was stopped, the enzyme was filtered off and the combined filtrates were concentrated by removing liquid in vacuo. Distillation of the residue gave (S)-1 ([α]²⁰₀ = -45°, c = 5, MeOH) and (R)-acetate 1 ([α]²⁰₀ = 114°, c = 2, MeOH). Chiral HPLC analysis showed that in all catalysed transesterification reactions with different enol esters only the (R)-1-alcohol was converted to (R)-1-ester.

The racemic esters were synthetically prepared by stirring 1.1 equivalent anhydride or acid chloride with 1 in pyridine for 4 hours. After evaporation, the product was purified by column chromatography or distillation resulting in a pure racemic compound as reference for HPLC.
Enantioselective aminoacylase-catalysed transesterification of secondary alcohols

Table 4.5: HPLC conditions of the acylation of 1-phenylethanol: solvent composition and retention times

<table>
<thead>
<tr>
<th>Acyl donor</th>
<th>Solvent</th>
<th>t&lt;sub&gt;r&lt;/sub&gt; ester</th>
<th>t&lt;sub&gt;r&lt;/sub&gt; alcohol</th>
<th>t&lt;sub&gt;s&lt;/sub&gt; alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>vinyl acetate</td>
<td>9/1</td>
<td>7.25</td>
<td>13.8</td>
<td>15.7</td>
</tr>
<tr>
<td>vinyl propionate</td>
<td>95/5</td>
<td>6.63</td>
<td>13.8</td>
<td>15.6</td>
</tr>
<tr>
<td>vinyl butyrate</td>
<td>95/5</td>
<td>6.47</td>
<td>13.8</td>
<td>15.6</td>
</tr>
<tr>
<td>vinyl acrylate</td>
<td>97/3</td>
<td>8.20</td>
<td>12.6</td>
<td>13.9</td>
</tr>
<tr>
<td>vinyl crotonate</td>
<td>97/3</td>
<td>9.10</td>
<td>12.6</td>
<td>13.9</td>
</tr>
<tr>
<td>vinyl hexanoate</td>
<td>96/4</td>
<td>7.13</td>
<td>31.7</td>
<td>41.0</td>
</tr>
<tr>
<td>vinyl octanoate</td>
<td>96/4</td>
<td>6.95</td>
<td>31.4</td>
<td>40.4</td>
</tr>
<tr>
<td>vinyl decanoate</td>
<td>98/2</td>
<td>6.55</td>
<td>20.0</td>
<td>24.7</td>
</tr>
<tr>
<td>vinyl benzoate</td>
<td>99/1</td>
<td>9.10</td>
<td>35.8</td>
<td>47.7</td>
</tr>
<tr>
<td>vinyl laurate</td>
<td>98/2</td>
<td>7.36</td>
<td>30.4</td>
<td>41.3</td>
</tr>
</tbody>
</table>

Enzyme catalysed acylation of alcohols with vinyl acetate

A mixture of 0.8 mmol (±) alcohol 1-12 and 1.2 mmol vinyl acetate was dissolved in 5 ml n-hexane containing 50 U aminoacylase (5 mg protein). The course of the reaction was followed by chiral HPLC (see Table 4.6) using cumene as the internal standard. When the reaction was complete or after 7 days the enzyme was filtered off and the combined filtrate were evaporated. The compounds 1-12 were purified from the residues by silica gel flash chromatography (EtOAc/hexane 10 / 90) to afford (S)-alcohol and (R)-ester.

The absolute configuration of the substrates have been assigned by measuring the optical rotation: (S)-1 ([α] = -45°, c = 5, MeOH) and (R)-acetate 1 ([α] = +114°, c = 2, MeOH); (S)-2 ([α] = -28°, c = 1, MeOH) and (R)-acetate 2 ([α] = +100°, c = 1, CHCl₃); (R)-6 ([α] = +20°, c = 1, MeOH) and (S)-acetate 6 ([α] = -10°, c = 1, CHCl₃); (S)-7 ([α] = -30°, c = 1, MeOH) and (R)-acetate 7 ([α] = +94°, c = 1, CHCl₃); (S)-8 ([α] = -25°, c = 2.2, MeOH) and (R)-acetate 8 ([α] = +33°, c = 1, CHCl₃); (S)-9 ([α] = -76°, c = 2.2, MeOH) and (R)-acetate 9 ([α] = +45°, c = 1, CHCl₃); (S)-10 ([α] = -34°, c = 1, CHCl₃) and (R)-acetate 10 ([α] = +45°, c = 1, CHCl₃); (S)-11 ([α] = -28°, c = 1, CHCl₃) and (R)-acetate 11 ([α] = +51°, c = 1, CHCl₃). Based on chiral HPLC, the retention time of the other enantiomer was compared with those substrates to determine their configuration. The optical rotation of 3 was determined by comparing the retention time of the enantiopure substrates which were commercially available.

The racemic products were synthetically prepared by stirring 1.1 equivalent acetic anhydride with alcohol in pyridine for 6 hours. After removing the pyridine and excess of acetic anhydride by evaporation the products were purified by column chromatography. The racemic
product was used as a reference for the HPLC. The prepared S-ester was not shown in the HPLC chromatograms of enzymatic catalysed reactions, which indicates the chiral discrimination of the catalyst. The transesterification of 1 with vinyl acetate in different solvents was carried out in the same manner as in hexane. Before injection all the samples were diluted with hexane/isopropanol mixture (1:1).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Solvent Hexane/2-propanol (v/v)</th>
<th>t_r ester (min)</th>
<th>t_r alcohol (min)</th>
<th>t_s alcohol (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/1</td>
<td>7.25</td>
<td>13.8</td>
<td>15.7</td>
</tr>
<tr>
<td>2</td>
<td>95/5</td>
<td>8.53</td>
<td>32.1</td>
<td>37.7</td>
</tr>
<tr>
<td>3a</td>
<td>97/3</td>
<td>8.17</td>
<td>32.1</td>
<td>35.0</td>
</tr>
<tr>
<td>4</td>
<td>98/2</td>
<td>9.21</td>
<td>24.7</td>
<td>28.2</td>
</tr>
<tr>
<td>5</td>
<td>98/2</td>
<td>8.40</td>
<td>13.6</td>
<td>15.0</td>
</tr>
<tr>
<td>6</td>
<td>99/1</td>
<td>11.4</td>
<td>55.2</td>
<td>98.2</td>
</tr>
<tr>
<td>7</td>
<td>99/1</td>
<td>6.79</td>
<td>14.0</td>
<td>17.7</td>
</tr>
<tr>
<td>8</td>
<td>95/5</td>
<td>25.1</td>
<td>71.4</td>
<td>59.0</td>
</tr>
<tr>
<td>9</td>
<td>95/5</td>
<td>9.44</td>
<td>37.6</td>
<td>22.9</td>
</tr>
<tr>
<td>10</td>
<td>98/2</td>
<td>9.06</td>
<td>42.4</td>
<td>49.9</td>
</tr>
<tr>
<td>11</td>
<td>99/1</td>
<td>8.64</td>
<td>32.9</td>
<td>36.9</td>
</tr>
<tr>
<td>12</td>
<td>99/1</td>
<td>11.0</td>
<td>48.9</td>
<td>51.0</td>
</tr>
</tbody>
</table>

a: chiralcel OB column was used

References


Enantioselective aminoacylase-catalysed transesterification of secondary alcohols


Chapter 4


Enantioselective aminoacylase-catalysed transesterification of secondary alcohols


Efficient transesterifications mediated by surfactant-conjugates of aminoacylases

Abstract
Surfactant conjugates of aminoacylase (E.C. 3.5.1.14) were prepared by colyophilisation of the crude enzyme with poly(ethylene glycol), poly(vinyl pyrrolidone), diethylene glycol monocetyl ether or Aerosol OT (AOT). The resulting enzyme-conjugates were insoluble in organic medium and highly efficient in the enantioselective acylation of 1-phenylethanol with vinyl acetate or butyrate (E>500). In a medium with only reactants and products as solvent the reaction rate enhancement relative to the native enzyme was up to 400. A space-time yield of 2000 g L$^{-1}$ d$^{-1}$ of (R)-1-phenylethylbutyrate was achieved using the aminoacylase-AOT conjugate, which makes this reaction potentially attractive for industrial application.
Efficient transesterifications mediated by surfactant-conjugates of aminoacylases

Introduction

Aminoacylase (E.C. 3.5.1.14) from *Aspergillus* species has considerable synthetic potential. It is used, for example, in the industrial synthesis of L-amino acids by enantioselective deacylation (see Chapter 1). In anhydrous organic media, aminoacylase acts as a transesterification catalyst with remarkable enantioselectivity and scope of several substrates. In Chapter 4 we have shown that a wide variety of aryl aliphatic alcohols is acylated by aminoacylase in the presence of vinyl alkanoic esters. These applications involved the use of crude enzyme isolates and we surmised that a modest effort at biocatalyst optimisation could result in a considerably improved efficiency.

In this context our attention was drawn to enzyme surfactant conjugates. Solubilisation of lipases and proteases in organic media via conjugation with various surfactants has been demonstrated. Conjugation can involve anionic, non-ionic and covalent bonding. Four different methodologies have been used for conjugating enzymes and amphiphilic modifiers: extraction, precipitation, colyophilisation and modification (covalent bonding). The first method is based on extraction of the enzyme into an organic phase containing a suitable counterion. Reversed micelles or micro emulsions have been prepared in a similar way. Surfactant coated-enzymes have been obtained as precipitates by mixing aqueous solutions of the enzyme and an surfactant conjugate. Colyophilisation, the easiest and fastest method to prepare enzyme-conjugates, is based on removing the water from an enzyme solution containing buffer salts and surfactants. Increased enzyme activity and enantioselectivity were obtained by colyophilisation of enzymes with amphiphilic compounds, such as crown ethers and β-cyclodextrin. Finally, the covalent modification of enzymes usually involves a nucleophilic reaction of amino groups at the surface of the protein with activated surfactants or polymers.

We adopted the colyophilisation technique to prepare surfactant conjugates of aminoacylase, because this technique is readily compatible with anhydrous organic solvents and easy to prepare. In this chapter we report that colyophilisation of aminoacylase with different surfactants considerably enhances the catalytic activity of the resulting preparation. To demonstrate the efficiency of this immobilization method, aminoacylase-surfactant conjugate catalysed transesterification reactions of 1-phenylethanol (Figure 5.1) were compared with those catalysed by lipases.

![Figure 5.1: Transesterification of 1-phenylethanol (1) with vinyl esters](image-url)

Figure 5.1: Transesterification of 1-phenylethanol (1) with vinyl esters
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Results and discussion

Lyophilisation method

The protein content of the commercial aminoacylases (E.C. 3.5.1.14) from *Aspergillus melleus* and *A. oryzae* was determined with a standard Bradford assay. Both preparations contained only a small amount of protein: 45 and 85 mg protein per g of solid, respectively. In the standard activity test, the hydrolysis of N-acetyl-l-methionine, both preparations had essentially the same activity, viz., 10 and 11 U per mg protein, respectively.

Table 5.1: Properties of the commercial aminoacylases from *A. melleus* and *A. oryzae*

<table>
<thead>
<tr>
<th>Source</th>
<th>Amount of protein (mg (g solid)^{-1})</th>
<th>Activity (U (mg protein)^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. melleus</em> (native)</td>
<td>45</td>
<td>10.0</td>
</tr>
<tr>
<td><em>A. oryzae</em> (native)</td>
<td>85</td>
<td>11.2</td>
</tr>
</tbody>
</table>

Conjugates of the aminoacylase from *A. melleus* with the non-ionic surfactants Brij 52 (diethylene glycol monocetylether), PEG (polyethylene glycol) and PVP (polyvinylpyrrolidone), as well as with the anionic Aerosol OT (sodium bis(2-ethylhexyl)sulfosuccinate: AOT)\(^{17}\) were prepared by colyophilisation. For comparison the enzyme was also modified by covalent attachment to activated PEG (see Table 5.2). The protein contents of the preparations were recalculated from the amounts of native enzyme and the increase in dry weight of the enzyme-surfactants conjugates after lyophilisation. Accordingly, the preparations of aminoacylase from *A. melleus* should contain at least 7.5 mg of protein per g lyophilsate (11.8 mg protein per g for AOT). As shown in Table 5.2 this is indeed observed, with the exception of the Brij 52 conjugate, which had a much higher total weight and correspondingly lower protein content. Presumably this preparation contains tightly bound water that is not removed by lyophilisation.

Lyophilising the native enzyme preparations from aqueous solutions followed by rehydration caused considerable deactivation (Table 5.2), which led to a loss of 66% of the original activity of the *A. melleus* enzyme and 86% for the one from *A. oryzae*. Colyophilisation with an amphiphilic compound partially suppressed the deactivation of the aminoacylases. The lyoprotecting effect on the *A. melleus* aminoacylase was most pronounced with Brij 52. The retained activity of the aminoacylase from *A. oryzae* upon colyophilisation with PVP K30 or AOT was considerably less than that of the *A. melleus* enzyme. We note that the activity test is performed in aqueous medium, in which the surfactant and the enzyme are expected to dissociate. Therefore its predictive value for application in organic media is limited. Covalent modification by a reaction of activated-PEG and the aminoacylase from *A. melleus* resulted in a very considerable loss of activity (approx. 65%). A similar loss of activity has been found before in comparable systems.\(^{18}\)
Efficient transesterifications mediated by surfactant-conjugates of aminoacylases

Table 5.2: Hydrolytic activity of surfactant conjugates of aminoacylases

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein fraction (mg g⁻¹)</th>
<th>Retained activity (U (mg protein)^⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. melleus</em> conjugates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>45.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Brij 52</td>
<td>3.2</td>
<td>9.0</td>
</tr>
<tr>
<td>PEG 20.000</td>
<td>5.8</td>
<td>6.4</td>
</tr>
<tr>
<td>PEG 3400</td>
<td>5.4</td>
<td>5.9</td>
</tr>
<tr>
<td>PEG activated</td>
<td>6.1</td>
<td>3.7</td>
</tr>
<tr>
<td>PVP K30</td>
<td>6.7</td>
<td>6.2</td>
</tr>
<tr>
<td>AOT</td>
<td>10.8</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*A. oryzae* conjugates

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein fraction (mg g⁻¹)</th>
<th>Retained activity (U (mg protein)^⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>85.0</td>
<td>1.6</td>
</tr>
<tr>
<td>PVP K30</td>
<td>12.8</td>
<td>3.9</td>
</tr>
<tr>
<td>AOT</td>
<td>19.1</td>
<td>4.4</td>
</tr>
</tbody>
</table>

[a: the activity was measured after colyophilisation with buffer]

*Transesterification of 1-phenylethanol in organic solvent*

The native aminoacylases from *A. melleus* and *A. oryzae* acts highly enantioselectively (E>500) in the acylation of 1-phenylethanol (1) with different vinyl esters. The effect of the chain length of the acyl donors on the rate of the acyl transfer was studied for both enzymes. The catalysts showed optimum activity with vinyl butyrate (C=4). The *A. melleus* enzyme was twice as active as the one from *A. oryzae* for all acyl donors investigated. A longer chain length of the acyl donor resulted in a decrease of the reaction rate by a factor six with a correspondingly longer reaction time for complete conversion of (R)-1.

![Graph](image)

Figure 5.3: Effect of the chain length of the acyl donor on the transesterification rate (in μmol product per minute per mg of protein) of 1 catalysed by aminoacylase from *A. melleus* (▲) and aminoacylase from *A. oryzae* (□).
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With vinyl butyrate a 50% conversion of 1 was reached within 30 hours and (S)-1 and (R)-1 acetate were obtained in quantitative yield with both catalysts. Although the specific hydrolytic activity of the enzyme from A. oryzae was slightly higher (Table 5.1), the initial rate in the transesterification reaction was markedly higher for A. melleus aminoacylase.

The surfactant conjugates of the aminoacylases from both Aspergillus species were similarly applied in the transesterification of 1 (see Table 5.3). Contrary to expectation, the conjugates did not dissolve in the reaction medium but formed a separate (liquid) phase. The initial activity of the aminoacylase-conjugates, based on protein weight, was 4 to 57 times as high as that of the native enzyme although the colyophilisates had lost up to 50% of their original hydrolytic activity (see Table 5.2). In particular the AOT-conjugates of the aminoacylases were highly efficient transesterification catalysts. A quantitative conversion of (R)-1 was accomplished in 3 h in the presence of the A. oryzae aminoacylase AOT-conjugate and in 4.7 h with the one from A. melleus. When the amount of protein is taken into consideration the initial rates of the AOT-conjugates were more than 50 times higher than those of the native aminoacylase. As shown, the initial rate of the A. melleus aminoacylase conjugate is higher than those with A. oryzae, similar as for the native enzymes.

Table 5.3:

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Surfactant</th>
<th>Protein (mg)</th>
<th>Initial rate (mmol (g prot.)⁻¹ min⁻¹)</th>
<th>Time (h)</th>
<th>Conv. (%)</th>
<th>Retained act. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. melleus</td>
<td>None</td>
<td>4.5</td>
<td>0.11</td>
<td>28</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Brij 52</td>
<td>0.32</td>
<td>1.34</td>
<td>72</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>PEG 20.000c</td>
<td>0.58</td>
<td>0.71</td>
<td>120</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>PVP K30</td>
<td>0.67</td>
<td>0.44</td>
<td>168</td>
<td>42</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>AOT</td>
<td>1.08</td>
<td>6.30</td>
<td>4.7</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>A. oryzae</td>
<td>None</td>
<td>8.5</td>
<td>0.05</td>
<td>30</td>
<td>50</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>PVP K30</td>
<td>1.3</td>
<td>0.24</td>
<td>168</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>AOT</td>
<td>1.9</td>
<td>5.20</td>
<td>3</td>
<td>50</td>
<td>24</td>
</tr>
</tbody>
</table>

a. reaction conditions: 100 mg catalyst, 0.8 mmol 1, 1.2 mmol vinyl butyrate in 5 ml hexane
b. the activity was measured after 7 days in hexane (U_out/U_in); whereas U_in is determined as the enzyme activity before the reaction and U_out after the reaction.
c. the initial rates with activated-PEG- and PEG 3400-aminoacylase conjugates were not determined
Efficient transesterifications mediated by surfactant-conjugates of aminoacylase

The surfactant-enzyme conjugates did not, however, fully maintain their high initial activity, as shown by the reaction times required for full conversion of (R)-1. These were 2 to 4 times longer than would be expected on the basis of protein contents and initial transesterification activity. It would seem that the enzyme-conjugates gradually lose activity under the reaction conditions. To test this hypothesis, the activity of the enzyme preparations was measured after use. After 7 days in anhydrous hexane and subsequent rehydration the native enzymes from both Aspergillus species had lost 60 % of their original hydrolytic activity. We conclude that the structure of the enzyme changes irreversibly under the reaction conditions. The activity loss of the enzyme-conjugates after 7 days in hexane, which was 75 % to 85% of the original hydrolytic activity (see Table 5.2), was much more severe. This amounts to a net destabilising effect of the surfactants, whereas stabilisation would be expected based on previous literature.\textsuperscript{17,19,20} In a reaction medium with only substrate and product as solvent the loss of activity of the enzyme-conjugate was remarkably lower (see next section).

The structure of the enzyme conjugates is rather a matter of conjecture. It would be expected that the surfactant molecules are randomly stacked around the enzyme surface. The retained activity of the AOT-conjugates was almost twice that of the other surfactant-aminoacylase conjugates. Apparently the AOT-anions are more efficient at stabilising the enzyme than the neutral amphiphiles, which can only form hydrogen bonds.

\textit{Transesterification of 1-phenylethanol in a solvent free reaction medium}

Solventless reaction systems are often used in combination with lipases due to the improved performance and simple work-up. We performed solvent free transesterifications using a small excess of vinyl acetate as the acyl donor for easy comparison of our results to those obtained with lipases.\textsuperscript{21,22} The enzyme conjugates did not dissolve in this reaction system but formed a second liquid phase, similar to the reactions in hexane (see earlier). The conjugates with Brij 52, PEG 20.000 and PVP formed apparently transparent emulsion upon agitation, which separated into two liquid phases upon standing.

The acylation initial rates of 1 with vinyl acetate in hexane were 53 and 23 μmol min\(^{-1}\) (g protein\(^{-1}\)) for aminoacylase from A. melleus and A. oryzae species, respectively (see previous section). In a solvent free medium the rates were increased by a factor 3 to 4 (see Table 5.4). The reaction times required for full conversion were longer than in previous experiments because the substrate/catalyst ratio was 15 times higher. Approximately 30 % conversion was reached after one week with the native aminoacylases. Complete conversion of (R)-1 was achieved in the presence of the conjugates with AOT, Brij and PEG 20.000. The reaction times were 1,5-4 times as long as would be estimated from the initial rates, strongly indicating that the biocatalysts slowly lose activity.

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The colyophilisation of aminoacylase from A. melleus with PEG 3400 resulted in an enzyme-PEG-conjugate that was much less active than the one with PEG 20.000. A similar effect of the molecular weight of the surfactant on the catalytic activity in organic media has been reported previously.\textsuperscript{25} Covalent modification of the aminoacylase from A. melleus with PEG gave an inefficient transesterification catalyst. Its initial activity was low compared with the physical PEG-conjugates, although higher than that of the native enzyme. The low final conversion (7% in 7 days) strongly indicates that the catalyst is rapidly deactivated under the reaction conditions.

The best result in the solventless transesterification was obtained with the AOT-conjugate of the aminoacylase from A. oryzae. Within 20 hours (R)-1 was completely converted, which resulted in a space-time yield of 435 g L\(^{-1}\) d\(^{-1}\). In contrast, with the AOT-conjugate of the A. melleus enzyme 72 hours were necessary to obtain 50% conversion with a STY of 122 g L\(^{-1}\) d\(^{-1}\). The STY for the AOT-conjugate of A. oryzae was a factor 7 higher than that of the ChiroCLEC-PC of Pseudomonas cepia lipase. The productivity, on the other hand, was almost 8 times lower and we note that solvent was added in the case of lipases (which reduces the STY by a factor of 2).

Table 5.4: Aminoacylase catalysed transesterification of 1-phenylethanol with vinyl acetate\textsuperscript{a}

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Surfactant</th>
<th>Initial rate</th>
<th>Conv.</th>
<th>Time</th>
<th>STY</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mmol (g prot.(^{-1}) min(^{-1}))</td>
<td>(%)</td>
<td>(h)</td>
<td>(g L(^{-1}) d(^{-1}))</td>
<td>(g (g prot.(^{-1}) h(^{-1}))</td>
<td></td>
</tr>
<tr>
<td>A. melleus</td>
<td>None\textsuperscript{b}</td>
<td>0.18</td>
<td>33</td>
<td>168</td>
<td>35</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Brij 52</td>
<td>3.0</td>
<td>50</td>
<td>168</td>
<td>51</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>PEG 20.000</td>
<td>3.5</td>
<td>50</td>
<td>120</td>
<td>72</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>PEG 3400</td>
<td>1.0</td>
<td>10</td>
<td>168</td>
<td>10</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>PEG act.</td>
<td>0.5</td>
<td>7</td>
<td>168</td>
<td>7</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>PVP K30</td>
<td>1.6</td>
<td>35</td>
<td>168</td>
<td>37</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>AOT</td>
<td>4.9</td>
<td>50</td>
<td>72</td>
<td>122</td>
<td>13.3</td>
</tr>
<tr>
<td>A. oryzae</td>
<td>None\textsuperscript{b}</td>
<td>0.09</td>
<td>27</td>
<td>168</td>
<td>28</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>PVP K30</td>
<td>0.36</td>
<td>33</td>
<td>168</td>
<td>35</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>AOT</td>
<td>7.4</td>
<td>50</td>
<td>20</td>
<td>435</td>
<td>25.2</td>
</tr>
<tr>
<td>Lipase\textsuperscript{c}</td>
<td>None</td>
<td>n.d.</td>
<td>50</td>
<td>48</td>
<td>27</td>
<td>0.1</td>
</tr>
<tr>
<td>CLEC\textsuperscript{d}</td>
<td>None</td>
<td>n.d.</td>
<td>50</td>
<td>16</td>
<td>62</td>
<td>197.4</td>
</tr>
</tbody>
</table>

a. reaction conditions: 200 mg catalyst, 24.6 mmol 1, 27 mmol vinyl acetate.
b. enzyme used without colyophilisation.
c. native enzyme from Pseudomonas species; reaction conditions: 200 mg catalyst, 10 mmol 1, 30 mmol vinyl acetate in 15 ml tBuOMe.\textsuperscript{21}
d. reaction conditions: 1.3 mg ChiroCLEC-PC, 50 mmol 1, 50 mmol vinyl acetate and 100 ml toluene.\textsuperscript{22}
Efficient transesterifications mediated by surfactant-conjugates of aminoacylases

We attempted to improve the productivity of our catalyst system by colyophilising semi-purified aminoacylases from both sources with surfactants but we obtained enzyme preparations with much lower catalytic activity (data not shown) than that of conjugates prepared from the crude enzyme. Apparently, the adjuvants presents in the crude preparations a stabilising effect on the protein, as suggested previously.\textsuperscript{10,11} In addition we measured the stability of the aminoacylase AOT-conjugates by repetitive batch reactions. After 5 cycles of the AOT-conjugates catalysed acylation of 1-phenylethanol with vinyl acetate, the productivity of both catalysts was decreased by 25% compared with the first cycle (see Table 5.4; 13 and 25 g (g prot\textsuperscript{-1} h\textsuperscript{-1}, respectively).

The effect of the pH was briefly investigated over the pH 5-9 range. We found that colyophilisation at pH 8 (100mM phosphate buffer) resulted in optimum transesterification activity. It should be noted that the buffer is an absolute requirement; colyophilisation from demineralised water without additional buffer resulted in preparations with very low activity.

As shown in the previous section aminoacylases from both \textit{Aspergillus} species have optimum acyl transfer activity towards 1 when vinyl butyrate is the acyl donor. A very similar effect - an increase in initial rate by a factor 2-4, compared with vinyl acetate - was observed with most conjugates (compare Table 5.4 and 5.5).

<p>| Table 5.5: Aminoacylase catalysed transesterification of 1-phenylethanol with vinyl butyrate\textsuperscript{a} |
|----------------|----------------|-------|---------|----------|-----------|</p>
<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Surfactant</th>
<th>Initial rate</th>
<th>Conv.</th>
<th>Time</th>
<th>STY</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mmol (mg prot\textsuperscript{-1} min\textsuperscript{-1})</td>
<td>(%)</td>
<td>(h)</td>
<td>(g L\textsuperscript{-1} d\textsuperscript{-1})</td>
<td>(g (g prot\textsuperscript{-1} h\textsuperscript{-1})</td>
</tr>
<tr>
<td>\textit{A. melleus}</td>
<td>None\textsuperscript{b}</td>
<td>0.4</td>
<td>47</td>
<td>168</td>
<td>47</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Brij 52</td>
<td>6.9</td>
<td>50</td>
<td>168</td>
<td>51</td>
<td>22.1</td>
</tr>
<tr>
<td></td>
<td>PEG 20.000</td>
<td>15.3</td>
<td>48</td>
<td>50</td>
<td>175</td>
<td>64.4</td>
</tr>
<tr>
<td></td>
<td>PVP K30</td>
<td>3.6</td>
<td>168</td>
<td>50</td>
<td>50</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>AOT</td>
<td>20.3</td>
<td>22</td>
<td>50</td>
<td>383</td>
<td>41.1</td>
</tr>
<tr>
<td>\textit{A. oryzae}</td>
<td>None\textsuperscript{b}</td>
<td>0.2</td>
<td>45</td>
<td>168</td>
<td>45</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>PVP</td>
<td>2.4</td>
<td>120</td>
<td>50</td>
<td>70</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>AOT</td>
<td>74.1</td>
<td>4</td>
<td>50</td>
<td>2104</td>
<td>127.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} reaction conditions: 200 mg catalyst, 24.6 mmol 1, 27 mmol vinyl butyrate
\textsuperscript{b} enzyme used without colyophilisation

All catalysts completely converted (R)-1 within 7 days and the STY increased accordingly. Two extremes are worth noting. (1) The effect of the donor on the conjugate of Brij 52 with \textit{A. melleus} enzyme was relatively modest. The initial rate of the acyl transfer for the Brij-52-
Chapter 5

conjugate of *A. melleus* was two times higher for vinyl butyrate (see Table 5.4 and 5.5), whereas the productivity and STY were comparable with vinyl acetate as acyl donor under similar reaction conditions. (2) The AOT-conjugate of the *A. oryzae* enzyme was a highly efficient transacylation catalyst with vinyl butyrate. Complete conversion of (R)-1 was accomplished within 4 h, which corresponds with a STY of 2 kg L\(^{-1}\) d\(^{-1}\) and a productivity of 127 g (g protein\(^{-1}\)) h\(^{-1}\).

Conclusions

Colyophilisation of the aminoacylases from *Aspergillus oryzae* and *Aspergillus melleus* with surfactants gave enzyme conjugates that were highly active in the enantioselective transesterification of racemic 1-phenylethanol. The best results were obtained with AOT as surfactant, in particular in a solvent free medium. Absolute enantioselectivity and enzyme stability was maintained throughout. In short, we have proved that surfactant-aminoacylase conjugates are suitable for application in the resolution of alcohols on an industrial scale.

Experimental procedure

*Materials*

Aminoacylase from *Aspergillus melleus* (E.C. 3.5.1.14) and Bradford reagent were purchased from Sigma. Aminoacylase from *Aspergillus oryzae* was a gift from Degussa (Hanau, Germany). Poly(vinyl pyrrolidone) K30 (PVP), Brij 52 (diethylene glycol monocetyl ether), Aerosol OT (sodium bis(2-ethylhexyl)sulfo succinate: AOT, 10% v/v), poly(ethyleneglycol) (PEG 3400), (R,S)-1-phenylethanol, vinyl decanoate and N-acetyl-l-methionine were obtained from Aldrich. Cumene, vinyl acetate and vinyl propionate were purchased from Acros, vinyl butyrate, poly(ethylene glycol) (PEG 20,000) and poly(ethylene glycol) mono-methyl ether were from Fluka. Vinyl hexanoate and vinyl octanoate were from Tokyo, Kasei, Kogyo Co, Ltd.

*Analysis and equipment*

The activity of aminoacylase preparations was measured using the standard hydrolytic assay of *N*-acetyl-l-methionine (see Chapter 2 and 3). One unit (U) will hydrolyse 1 µmol of *N*-acetyl-l-methionine per minute. Reversed phase HPLC analysis was performed using a custom-packed Symmetry C18 cartridge (Waters Radial-Pak, 8 x 100 mm, 7 µm) contained in a Waters RCM 8x10 compression unit, with detection on a Waters 486 tunable absorbance detector with Waters Millenium32 software. The products of *N*-acetyl-l-methionine hydrolysis were analysed using acetonitrile-phosphate buffer (50 mM; pH 2.2) 7.5:92.5 (v:v) as eluent (flow 1.5 ml/min for the first 4 minutes followed by 3 ml/min), with detection at 210 nm. The products of transesterification of 1-phenylethanol were analysed on chiral HPLC using Chiralcel OD column (Daicel Chemical Industries, Ltd., 250 x 4.6 mm), eluent flow 0.6 ml min\(^{-1}\), using cumene as internal standard, isopropanol-hexane 5:95 (v:v) as eluent and detected at 254 nm. Optical
Efficient transesterifications mediated by surfactant-conjugates of aminoacylases

rotations were measured using a Perkin Elmer 241 polarimeter. UV measurements were performed on a Cary 3 spectrophotometer from Varian.

**Enzyme lyophilisation**

To prepare surfactant coated protein 5 g dry weight of Brij 52, PEG 3400, PEG 20.000 or PVP k30 was dissolved in 50 ml buffer (100 mM KH₂PO₄, pH 8.0) and mixed with 1 g of enzyme. In the case of Aerosol OT, 28 g of emulsion (10% Aerosol) was mixed with 50 ml buffer (100 mM KH₂PO₄, pH 8.0). When the solution was homogeneous it was frozen in a rubber sealed freeze-dry flask (Salm en Kipp bv, 300 ml) at −40 °C and lyophilised for 16 hours.

The PEG-modified enzyme complexes were prepared by shaking 0.5 g of enzyme, 2.5 g of cyanuric chloride activated PEG⁵ in 50 ml buffer (100 mM KH₂PO₄, pH 8.0 and pH 9.0) for 1 hour. As with the other surfactant preparations the liquid was frozen and lyophilised.

**Transesterification reactions**

Experiments to investigate the effect of the chain length of the acyl donor on the transesterification rate were performed by shaking 100 mg of 1-phenylethanol (0.8 mmol) with 1.5 equivalent acyl donor (n = 0,1,2,3,5,7,9) (1.2 mmol), 100 mg catalyst and 5 ml hexane at room temperature (see Figure 5.2). During the first 5 hours the initial rate was measured by following the course of the reaction with chiral HPLC. When the conversions were higher than 15 % within 5 hours, the reaction was repeated and samples were taken every 15 minutes during the first 2 hours.

Transesterifications were also performed in the absence of solvent, by shaking 200 mg of surfactant-prepared catalyst in 3 g 1-phenylethanol (24.6 mmol) and 1.1 equivalent vinyl acetate. The sample for analysis were centrifuged to remove the surfactant layer from the substrate/product. The course of the reaction was followed by chiral HPLC. In the production of 1-phenylethyl butyrate 1.1 equivalent vinyl butyrate (27 mmol) was used instead of vinyl acetate. After 7 days the reaction mixture was centrifuged and the catalyst was removed from the liquid. After work-up a yield of 42 % of pure ester 1-(R) was achieved (optical rotation: +1-(R): [α]₀²⁰ =+81.8 (CHCl₃, c=1.2);-1-(S): [α]₀²⁰ =-45.0 (CHCl₃; c=1.0). The surfactant-coated complex was washed with hexane (3 x 50 ml) and dried under air. The loss of activity of the catalyst was measured using the hydrolytic activity test.

**References**

Chapter 5


Efficient transesterifications mediated by surfactant-conjugates of aminoacylases


Chapter 6

Immobilisation of thermolysin

Abstract

Thermolysin from Bacillus thermoproteolyticus rokko (E.C. 3.4.24.4) was immobilised in order to increase its operational stability. Two immobilisation methods were used, viz., covalent bonding on Eupergit C and cross-linking of enzyme crystals. In order to circumvent the unreliability of the classical protease activity assay (digestion of casein) a new, improved method was developed based on the hydrolysis of 3-(2-furylacryloyl)-glycly-L-leucine. Finally, the stability and operational life of the immobilised thermolysin was measured in the ammonolysis of Z-Phe-Ala-OMe yielding the amide derivative.
Immobilisation of thermolysin

Introduction

In nature many reactions are catalysed by metallo-enzymes. Many of these reactions are synthetically useful reactions, such as redox, aldol and hydrolysis reactions.\(^1\) The metalloprotease thermolysin (E.C. 3.4.24.4), for example, is used in the industrial synthesis of Z-L-Asp-L-Phe-OMe, a precursor of the artificial sweetener aspartame.\(^2\)\(^-\)\(^5\) (Figure 6.1)

![Chemical reaction diagram](image)

**Figure 6.1: Synthesis of Z-L-Asp-L-Phe-OMe**

Thermolysin is produced by *Bacillus thermoproteolyticus* and excreted in the culture broth.\(^6\) It is a thermostable neutral metalloprotein that belongs to the family of the endopeptidases and cleaves specifically peptide bonds in which the nitrogen is contributed by an amino acid residue with a hydrophobic side chain.\(^7\) Thermolysin has a monomeric structure with a molecular weight of 34.6 kDa.\(^8\)\(^,\)\(^9\) It consists of two domains, one a mainly β pleated sheet and the other mainly α-helical, with the active cleft, containing the catalytic Zn-ion, between the domains. Four calcium ions stabilise the structure.\(^10\) Because we wished to use thermolysin in anhydrous organic media, immobilisation was necessary to stabilise the enzyme.\(^11\)\(^,\)\(^12\) Moreover, it was our purpose to modify the catalytic properties of thermolysin by substitution of the metal atom (see Chapter 7) and immobilisation would facilitate the enzyme handling. Furthermore, to prevent changes of the enzyme structure during substitution it was deemed necessary to achieve a stable active site. A strong bonding with the carrier, such as the covalent bonding method is expected to prevent protein unfolding during modification.

In the literature the immobilisation of thermolysin by physical adsorption or ionic bonding\(^3\)\(^-\)\(^5\)\(^,\)\(^11\) has been described. We investigated the crosslinking of enzyme crystals\(^13\)\(^-\)\(^15\) as well as covalent attachment to oxirane acrylic beads (Eupergit C). Both are multipoint-attachment techniques that we expected to have an optimum stabilising effect. The methods were compared with regard to the recovered hydrolytic activity. Finally, the increased stability of the prepared immobilised batches was assessed on the basis of their performance in organic media. The activities of native and immobilised thermolysin in ammonolysis reactions were measured and compared.
Chapter 6

Results and discussion

Methods for determining enzyme activity

The proteolytic activity of the thermolysin preparations was measured by the hydrolysis of a peptide, casein, as well as 3-(2-furylacryloyl)-glycyl-L-leucine amide (FAGLA). Casein is built from 4 subunits with a molecular weight of approximately 20 kDa.\textsuperscript{16} It is commonly used to assay neutral proteases.\textsuperscript{17,18} The second method for determining the proteolytic activity of thermolysin was based on the hydrolysis of FAGLA, which is more specific for thermolysin.

The hydrolysis of casein was monitored by the spectrophotometric detection of tyrosine with Folin Ciocalteu’s reagent.\textsuperscript{19} The progress of the reaction was fitted to zero-order kinetics. The liberated amount of tyrosine was calculated from the calibration curve (Figure 6.2.). The activity of the native thermolysin in the hydrolysis of casein was 119 cU/mg. One unit (cU) will liberate one \( \mu \)mol tyrosine per minute.

![Graph showing calibration curve absorbance versus the concentration of tyrosine](image)

Figure 6.2: Calibration curve absorbance versus the concentration of tyrosine

The method, the digestion of casein detected with Folin Ciocalteu’s reagent, has been applied to many proteases,\textsuperscript{16,19} although we think that it is not suitable for thermolysin. The structure is rather flexible\textsuperscript{20} but the high molecular weight (20 kDa) could cause some diffusion problems with the catalyst (Mw 34 kDa). Furthermore, casein contains phosphate groups which are known as inhibitors for thermolysin.\textsuperscript{21,22} Besides that, casein is preferably soluble in phosphate buffer, which also increases the concentration of phosphate groups in the medium. Moreover, it is generally known that phosphate groups can extract essential calcium atoms from thermolysin, which decreases its stability. Finally the casein assay, the reliability of the reagent which is a combination of phosphomolybdic and phosphotungstic acid complexes that react with Cu\(^+\) generated from Cu\(^{2+}\) by cysteine or the phenols and indoles of tyrosine and tryptophan, could be influenced by the presence of sugars, detergents and certain buffers. Thermolysin contains, according to the Bradford assay, more than 90% salts and adjuvants used for stabilisation during freeze-drying, which could interfere with the assay.
Im mobilisation of thermolysin

The hydrolysis of FAGLA was developed by Feder.\textsuperscript{23} The method is based on the hydrolysis of a dipeptide blocked with a furylacryoyl group which can easily be monitored by spectrometry (See Figure 6.3). The proteolytic activity was measured by monitoring the absorption during 3 minutes. The progress of the reaction was analysed according to pseudo-first order kinetics by fitting the absorption to the function $K_2 \cdot e^{-kt}$ (see appendix).

\begin{equation}
Y = [S]_0 e^{-kt}
\end{equation}

![Chemical structure](image)

Figure 6.3: Amounts of 3-(2-furylacryoyl)glycine formed by the hydrolysis of FAGLA by the native enzyme (bottom curve) and the cross-linked enzyme (Pepti-CLEC\textsuperscript{TM}-TR) (top curve). Circles represent experimental datapoint and the line show the fit of the equation. ($Y= (A-A_{\infty})/\Delta A$; see also appendix)

Native thermolysin led to complete conversion of the substrate within 1.5 minutes whereas a similar amount of cross-linked enzyme crystals (CLECS) required at least 3 minutes. In the assay of the native enzyme the substrate concentration seems to start at a lower point due to the loss of data points during the time period of mixing substrate and catalyst followed by placing the sample in the UV spectrophotometer (few seconds). The fluctuations in the data points of the CLC
sample are caused by the presence of solid catalyst particles in the aqueous medium. In both cases the data points closely correspond with pseudo-first order kinetics (see Figure 6.3; solid line). Using this assay an activity of 2800 fU/mg was observed for the native enzyme and 347 fU/mg for the Pepti-CLEC™-TR (One unit (fU) will hydrolyse one μmol FAGLA per minute). We note that due to the pseudo first-order kinetics it is essential to monitor the reaction over time instead of taking one data-point. Surprisingly, this notion has generally been disregarded in the literature.⁵⁴

**Synthesis of thermolysin crosslinked enzyme crystals**

To prepare crosslinked enzyme crystals we first focussed our efforts on the preparation of protein crystallisation. Five different crystal shapes of thermolysin are known.⁸,¹³ They have been prepared by variation of the salts, temperature and protein concentration. We have crystallised thermolysin according to the procedure outlined in Scheme 6.1.

![Scheme 6.1: Preparation of thermolysin crystal shapes](image)

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Immobilisation of thermolysin

We observed only three different crystals shapes (Figure 6.4): needles (a), the rice shape (b) and the hexagonal rod (c). Needles and hexagonal rods break easily during shaking, with a consequent loss in activity and, hence, the rice shape is the preferred crystalline form of thermolysin.\textsuperscript{14,15}

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{crystals.png}
\caption{Different prepared crystal shapes of thermolysin}
\end{figure}

Unfortunately, while washing the crystals with buffer solutions to remove residual solutes, the crystals redissolved. When cross-linking the crystals with glutaraldehyde redissolution also occurred, due to the change of polarity and buffer concentration. We have attempted to make CLCS by varying the enzyme concentration, pH, solvent and buffer. So far we succeeded in the preparation of needle-shaped CLCS, which were prepared without washing the crystals. Because of the large number of variables that must be optimised to synthesise CLCS efficiently, we decided to use the commercial Pepti-CLEC\textsuperscript{TM}-TR prepared by Altus Biologics Inc in our further work.

\textbf{Immobilisation of thermolysin on Eupergit C}

Thermolysin was covalently immobilised on two types of Eupergit: Eupergit C (approx. 150μm, macroporous, spherical) and Eupergit C 250 L (approx. 200μm, macroporous, spherical). They are known as stable polymers in organic solvent. Attachment of the protein takes place by nucleophilic reaction of lysine residues at the surface of the protein with oxirane groups on the carrier. The content of oxirane groups is different for each carrier (see Table 6.1).\textsuperscript{25}

\begin{table}[h!]
\centering
\caption{Properties of Eupergit C}
\begin{tabular}{lccc}
\hline
 & Eupergit C & Eupergit C 250 L \\
\hline
Content of oxirane groups (μmol/ g dry weight) & >600 & >200 \\
\hline
\end{tabular}
\end{table}

To optimise the immobilisation method of thermolysin, variations were made in salt concentration, pH, temperature, buffer and enzyme concentration. The excess of available
Chapter 6

epoxide groups was easily removed by shaking the prepared beads with a TRIS buffer for a few hours. The best preparation showed an activity of 0.2 cU/ mg for thermolysin immobilised on Eupergit C and 3.4 cU/mg bonded on Eupergit C 250 L. The activity was measured from the immobilised catalyst.

Comparison of the immobilisation methods

The retained activity or immobilisation efficiency was first measured by the hydrolysis of casein (see table 6.2). All immobilisation methods resulted in a loss of proteolytic activity. The thermolysin needles had a hydrolytic activity of 88 cU/mg and lost 40% of their activity upon cross-linking. The activity of the cross-linked needles was lower than that of the commercial preparation of Pepti-CLECS\textsuperscript{TM}-TR, which seems reasonable, because we used unpurified needle crystals for cross-linking. Moreover, our crosslinking method was not optimised.

The immobilisation efficiency of the preparation of Pepti-CLEC\textsuperscript{TM}-TR is 70% according to the literature\textsuperscript{26} based on the hydrolysis of 3-(2-furylacryloyl)-glycyl-L-leucine amide (FAGLA). Crystallisation of the enzyme causes a decrease in activity of 20% and a further loss of 10% is due to crosslinking. In the hydrolysis of casein we observed an immobilisation efficiency of 72%, which is comparable with the published result of the FAGLA assay (see page 78).

Table 6.2: Hydrolytic activity of casein and immobilisation efficiency of various immobilised thermolysins

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity (cU/mg)</th>
<th>Immobilisation efficiency ((U_{out}/U_{in}) * 100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermolysin (native)</td>
<td>119</td>
<td>100</td>
</tr>
<tr>
<td>Thermolysin needles</td>
<td>88</td>
<td>74</td>
</tr>
<tr>
<td>Needle-shaped thermolysin-CLCS</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>Pepti-CLEC\textsuperscript{TM}-TR</td>
<td>86</td>
<td>72</td>
</tr>
<tr>
<td>Thermolysin/ Eupergit C</td>
<td>12\textsuperscript{a}</td>
<td>10</td>
</tr>
</tbody>
</table>

\textsuperscript{a} the activity was corrected by a factor 3.6 for the water uptake of the carrier (1 gram of dry Eupergit gives approximately 3.6 g catalyst)

The immobilisation of thermolysin on Eupergit C likewise resulted in a partial loss of catalytic activity and the immobilised thermolysin contained only 10% of its original hydrolytic activity. Besides the covalent binding of the amino groups on Eupergit C, the polymer contains acrylate groups, which form hydrophobic interactions with the enzyme. This could cause conformational changes in the enzyme molecule, which would explain the decrease in activity.

To obtain a more reliable comparison of both immobilisation methods and their substrate acceptance the hydrolysis of FAGLA was also investigated. As already mentioned the substrates
Immobilisation of thermolysin

have different molecular weights and rates of hydrolysis as shown by their unit definitions. The hydrolytic activity of immobilised thermolysin preparations is presented in Table 6.3. Unfortunately it was not possible to measure the reaction with the needle-shape CLCS due to their fragility. Vigorous magnetic stirring is necessary during UV measurement to have a fast mixing of substrate during the first minutes, which easily breaks the enzyme-crystals and result in a loss of activity.

The retained activity of the Pepti-CLEC™-TR, according to this procedure, was much lower compared to the casein assay, as well as to the published results which were also based on the hydrolysis of FAGLA.26 In this case the activity assay with FAGLA was used in a completely different manner. For the Pepti-CLEC catalysed hydrolysis of FAGLA, samples were taken after 10 minutes, whereas the hydrolytic reaction of the native enzyme was already finished within 1.5 minute.24 As mentioned in the previous section and shown in Figure 6.3, for this activity test it is crucial to monitor the hydrolytic reaction during the time instead of measuring a single data point.

Table 6.3 Hydrolytic activity of FAGLA and immobilisation efficiency of various immobilised thermolysins

<table>
<thead>
<tr>
<th>Batch</th>
<th>Activity (fU/mg)</th>
<th>Immobilisation efficiency (U_out/U_in)*100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermolysin (native)</td>
<td>2800</td>
<td>100</td>
</tr>
<tr>
<td>Pepti-CLEC™-TR</td>
<td>347</td>
<td>12</td>
</tr>
<tr>
<td>Thermolysin/ Eupergit C</td>
<td>43a</td>
<td>1.5</td>
</tr>
<tr>
<td>Thermolysin/Eupergit C (optimised)</td>
<td>155\textsuperscript{b}</td>
<td>5.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} the activity was adjusted for water uptake by the carrier (factor 3.6); purified and concentrated enzyme was used for immobilisation

Presumably the loss of enzyme flexibility could cause the low immobilisation efficiency observed for the commercial Pepti-CLEC™-TR, as was also noticed by other workers.27 The preparation and growing of enzyme crystals starts with purification of the protein. Under defined pH, temperature and additives the growing process of the crystals requires a certain conformation of the enzyme molecule. In Table 6.3 the hydrolytic activity test showed a decrease of efficiency compared to the results presented in Table 6.2, indicating that FAGLA was a less active substrate than casein for the Pepti-CLEC. Possibly, the enzyme molecule is fixed in a conformation which is favourable for casein rather than FAGLA as substrate.

The activity of thermolysin immobilised on Eupergit C was 50\% lower than that of the commercial Pepti-CLEC™-TR. Nonetheless, when we compare both immobilisation methods regarding their production and prices then the immobilisation of thermolysin on Eupergit C seems very promising.
Chapter 6

The application of immobilised thermolysin in catalysed ammonolysis reactions

In spite of the low hydrolytic activity of the immobilised thermolysin preparations we used them in ammonolysis reactions (Figure 6.4) in order to measure their catalytic activity and stability in organic media. The conversion of Z-(L)-PheAlaOMe was monitored at 40°C and 60°C over 5 days in t-BuOH with NH₃ (see Table 6.3).

![Chemical structure](image)

Figure 6.4: Thermolysin catalysed ammonolysis reaction

In the absence of any catalyst 10 % of the substrate was converted during 5 days at 40°C. With the native enzyme the conversion did not significantly exceed that of the background reaction. The immobilised thermolysin on Eupergit C, on the other hand, gave 41 % conversion (31 % after subtraction of the background reaction) which is rather low compared to lipases.²⁸

Table 6.3: The conversions and retained activities of thermolysin catalysed ammonolysis reaction

<table>
<thead>
<tr>
<th>Batch</th>
<th>Temp. (°C)</th>
<th>Catalyst (mg)</th>
<th>Protein fraction (mg/g solid)</th>
<th>Conversion (%)</th>
<th>Retained act. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank reaction</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Thermolysin (native)</td>
<td>40</td>
<td>50</td>
<td>3.2</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>50</td>
<td>3.2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Pepti-CLEC™-TR</td>
<td>40</td>
<td>30</td>
<td>30.0</td>
<td>33</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>30</td>
<td>30.0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Thermolysin/ Eupergit C (optimised)</td>
<td>40</td>
<td>167</td>
<td>16.7</td>
<td>41</td>
<td>69</td>
</tr>
</tbody>
</table>

a: reaction conditions: 100 mg of substrate in 5 ml tBuOH saturated with ammonia were gently shaken at 40 or 60°C.
b: protein fraction was measured according to the Bradford assay for native and immobilised thermolysin on Eupergit C (wet) whereas for the Pepti-CLEC® purified protein was used.
c: the conversion was measured after 5 days
d: after 5 days in tert-butylalcohol the activity was measured and retained activity was calculated according to the FAGLA-assay (Uout/Uin).
Immobilisation of thermolysin

The reaction conditions always resulted in a loss of hydrolytic activity. The native biocatalyst was completely inactive after 5 days in tert-butyl alcohol in the presence of ammonia. The immobilised preparations were only partially deactivated after reaction at 40°C; but after 5 days at 60°C less than 20 % of the original activity was maintained. We conclude that the immobilised catalysts are considerably more stable.

The product from the reaction with thermolysin immobilised on Eupergit C contained some acid, which presumably was formed by reaction with the small amount of water present in the immobilised batch of Eupergit C (1 g dry weight gives 3.6 g wet catalyst). We attempted to solve this problem by drying the Eupergit preparation. However, after drying with P₂O₅ or stepwise with acetone only 1 and 3 % of the original activity was left. This result showed that it is better to use wet immobilised enzyme preparations.

The amount of protein per g of catalyst for both immobilised catalysts is different. The polymer-bonded enzyme contained 40 % less protein per gram of catalyst compared to Pepti-CLEC-TR whereas for the ammonolysis 20% higher conversion was obtained at 40°C. The higher conversions in this case could partly be explained by the low water contents of the enzyme bonded to the epoxy-groups. Furthermore, for thermolysin (and in general for all enzymes) it is known that a low amount of water increases the conversions in organic solvents. Moreover, the retained activity of thermolysin immobilised on Eupergit C was higher than the Pepti-CLECS, which makes this immobilisation method favourable for this reaction, although the results are insignificant compared with lipases.

Conclusions

An improved activity test has been developed for thermolysin. It is based on the hydrolysis of 3-(2-furylacryloyl)-glycyl-l-leucine amide, which is subsequently analysed using first-order kinetics.

Immobilisation of thermolysin on Eupergit C or by preparation of crosslinked enzyme crystals (Pepti-CLEC™-TR) causes a very considerable decrease of hydrolytic activity that could be due to conformational changes and loss of flexibility. Although the stability and operational life in ammonolysis was improved by immobilisation, the catalytic activity of thermolysin was low compared to lipases.

Experimental procedure

Materials

Thermolysin from Bacillus thermoproteolyticus rokko (E.C. 3.4.24.4) was a gift from DSM, Eupergit C was a gift from Röhm GmbH (Darmstadt, Germany). 3-(2-Furylacryloyl)-glycyl-l-leucine amide was purchased from Sigma, Pepti-CLEC™-TR from Altus Biologis Inc., Casein from
Chapter 6

Aldrich and 1,3-dimethoxybenzene from Acros. Folin-Ciocalteu’s reagent was obtained from Fluka.

Analysis and equipment

UV measurements, to follow the liberated amount of tyrosine from the digestion of casein detected with reagent and the hydrolysis of FAGLA, were performed on a Cary 3 spectrophotometer from Varian. The datapoints from the hydrolysis of FAGLA were fitted with RR-GRAPH (produces X-Y graphs of scientific data and functions; version 6.0; Reactor Research Foundation Delft University).

Z-Phe-Ala-OMe was analysed by reversed phase HPLC on a custom-packed Symmetry C18 cartridge (Waters Radial-Pak, 8 x 100 mm, 7 μm) contained in a Waters RCM 8x10 compression unit, with a Waters 486 tunable absorbance detector with Waters Millenium32 Software. The products were analysed using methanol/water/trifluoracetic acid (60:40:0.1 (v:v)) as eluent (flow 1.0), with detection at 215 nm and 1,3-dimethoxybenzene as internal standard. The protein content was determined according to the Bradford assay as described in Chapter 2.

The preparation and growing of thermolysin enzyme crystals

A 2.5 g crude enzyme preparation was dissolved in 45 ml CaAc buffer (10mM). The pH was adjusted slowly to 10 with NaOH (2M). After the catalyst was dissolved the pH was stepwise titrated to pH 8 with HCl (2M). Calcium acetate was added to a final concentration of 1.2 M. The volume was determined and 30% (v:v) of DMSO was added to the solution. Finally the enzyme solution was purified and concentrated to 100 mg of enzyme per ml by centrifugation with an Amicon ultramembrane (cut-off 10 kDa) tube.

Thermolysin was crystallised by the addition of 1 volume demineralized water to 1 volume concentrated (100 mg/ml) protein solution. The solution was briefly vortexed and stood overnight at room temperature. Following this procedure hexagonal rods were prepared, whereas 3 volumes of water with 1 volume concentrated enzyme gave the preferred rice shape. The needles were prepared by using a higher volume of water(5:1 water/concentrated enzyme). Using different temperatures, buffer concentrations or using the seeding technique can easily influence the crystal growing.

Activity test of thermolysin

The proteolytic activity of thermolysin was measured by digestion of casein described by Morihara.17 One ml of 2% casein solution (50mM KH₂PO₄, pH 7.4; solubilised during heating for at least one hour) was activated for 30 minutes at 40°C and mixed with 2 ml thermolysin enzyme preparation (± 2 mg of crude thermolysin, for immobilised thermolysin on Eupergit C 150 mg; 0.5 % CaCl₂; 50 mM TRIS, pH 7.5) and shaken at 40°C for 10 minutes. The reaction was stopped
Immobilisation of thermolysin

after adding 2 ml of 10% trichloroacetic acid. The mixture was incubated for 30 minutes at the same temperature to destroy the diluted protein. The precipitate was centrifuged off and 1 ml of aqueous was vortexed with 5 ml 10% Na₂CO₃. The liberated amount of tyrosine was detected by adding 1 ml of Folin-Ciocalteu’s reagent and incubated for 30 minutes. Finally the absorbance was measured at 670 nm. The amount of tyrosine was calculated from the prepared calibration curve, absorbance versus concentration tyrosine (Figure 6.2). Units (U) were defined in μmol tyrosine per min per mg of enzyme.

The hydrolysis of FAGLA, 3-(2-furylacryloyl)-glycyl-L-leucine amide, was measured according to the method developed by Feder.²³ In 100 ml TRIS buffer (50 mM pH 7.5, 10 mM CaCl₂) 76.6 mg of substrate (2.49 mM) was dissolved. In a quartz cuvet 3 ml of buffer substrate was mixed with 1-5 mg of catalyst (in case of immobilised thermolysin on Eupergit C 5-10 mg) for 3 seconds and immediately placed in the UV-spectrophotometer. During stirring the decrease of absorbance was measured at 345 nm. After fitting the datapoints to the curve with the function \[ [S] = k_2 \cdot e^{-k_1 t} \] the activity in U/mg catalyst could be calculated (see Appendix; \( k_2 \) stands for \( k \)). Units (U) were defined in μmol FAGLA min⁻¹ per mg of enzyme.

Immobilisation on Eupergit C

The screening of the optimised immobilisation batch on Eupergit C was prepared by varying the buffer and enzyme concentration. For HEPES buffer 50 ml solutions of 0.5 M, 1.0 M and 1.5 M containing 1 mM of CaCl₂ were mixed with respectively 100 mg, 200 mg and 300 mg thermolysin (concentrated and washed enzyme solutions prepared by dialysing) and 1 g of Eupergit C (or 250 l) was added. After 72 hours shaking at 4°C the 9 precipitates were filtered and washed three times (4 hours) with 100 ml of TRIS buffer (100mM, 0.5 % CaCl₂) to destroy unreacted epoxide groups.

Thermolysin catalysed ammonolysate reactions

Z-amino esters were prepared according to the literature.²⁷ In a 5 ml ammonia saturated t-butyl alcohol (12.5 mmol) 100 mg of ester was dissolved and 50 mg of thermolysin (or 30 mg CLEC-TR or 167 mg immobilised thermolysin on Eupergit C) was added to start the reaction. The mixture was shaken for 5 days at specified temperature (40 or 60°C) and the course of the reaction was followed by HPLC. Samples were prepared by taken 100 μl reaction mixture diluting with 700 μl MeOH and adding 1,3-dimethoxybenzene as internal standard. The blank reaction was performed without additional catalysts under similar conditions.
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Immobilisation of thermolysin

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Appendix to Chapter 6

_Catalytic activity of thermolysin in the hydrolysis of FAGLA: pseudo first-order kinetics_

The thermolysin catalysed hydrolysis of FAGLA can be treated a pseudo first-order reaction under the restriction that \([S]\) is much smaller than \(K_m\):

\[ [S]_0 \ll K_m \]

From the literature\(^2\) it is known that the \(K_m\) for the hydrolysis of FAGLA is \(3.0 \times 10^{-2} \text{ mM}\), therefore we used a substrate concentration \(([S]_0)\) of \(2.49 \times 10^{-3} \text{ mM}\).

Under pseudo first order conditions the rate of the reaction is given by:

\[ v = \left( \frac{k_{\text{cat}}}{K_m} \right) [E_0] [S] \]

with

\[ k = \left( \frac{k_{\text{cat}}}{K_m} \right) [E_0] \]

and \(v = -d[S]/dt\) this gives:

\[ d[S]/dt = -k [S] \]

Integration gives

\[ [S] = [S]_0 e^{-kt} \]  \( (1) \)

The rate of reaction is monitored by UV/VIS spectrometry. The absorption at \(t\) is the sum of the absorption of the substrate, the product and the background absorption of the (immobilised) enzyme:

\[ A = \varepsilon_p [P] + \varepsilon_s [S] + A_{\text{background}} \]

The concentration of the product is given by:

\[ [P] = [S]_0 - [S] \]

therefore

\[ A = \varepsilon_p ([S]_0 - [S]) + \varepsilon_s [S] + A_{\text{background}} \]

\[ = \varepsilon_p [S]_0 + (\varepsilon_s - \varepsilon_p) [S] + A_{\text{background}} \]

\[ = \varepsilon_p [S]_0 + \Delta \varepsilon [S] + A_{\text{background}} \]

at \(t = \infty, [S] = 0\) therefore

\[ A = A_\infty = \varepsilon_p [S]_0 + A_{\text{background}} \]

this gives

\[ A = \Delta \varepsilon [S] + A_\infty \]

thus

\[ [S] = (A-A_\infty)/\Delta \varepsilon \]  \( (2) \)

\( \Delta \varepsilon = 317 \text{ mM}^{-1} \) and \(A_\infty\) is measured after several hours.

Combination of \((1)\) and \((2)\) gives:

\[ (A-A_\infty)/\Delta \varepsilon = [S]_0 e^{-kt} \]

When \((A-A_\infty)/\Delta \varepsilon\) is plotted against \(t\), the curve is fitted with the curve fitting programme RRgraph, this result in the value of \(k\). From \(k\) the activity of the enzyme preparate is calculated:

\[ \text{Activity} = (-k K_m)/[E_0] \]

\( (k \text{ in min}^{-1}; K_m \text{ in mM} = \mu \text{mol ml}^{-1}; [E_0] = \text{mg ml}^{-1} \) which resulted in Activity: U/mg)
Chapter 7

Metal substitution of thermolysin and its application in oxygen transfer reactions

Abstract
Thermolysin (E.C. 3.4.24.4), a stable zinc protease, was modified by substitution of the metal atom. Zinc was replaced by Co$^{2+}$, Mn$^{2+}$, MoO$_4^{2-}$, SeO$_4^{2-}$, WO$_4^{2-}$. The hydrolytic activity of the enzyme decreased by 95% compared to the native enzyme as a result of metal substitution. To increase its proteolytic activity and facilitate handling of the immobilised thermolysin preparations, viz. on Eupergit C and clecs, were used. The restored activity of the reconstituted enzymes was optimised to 50% when thermolysin was attached to the polymer. Tungstate-thermolysin immobilised on Eupergit C acted as a catalyst in the sulfoxidation of several substrates. Fundamentally, the properties of thermolysin were changed from hydrolytic towards redox activity by metal substitution.
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Introduction

Chemical modification of the reactive group in the active site is a powerful tool for modifying the catalytic activity of enzymes. For example, subtilisin was transformed into selenosubtilisin, which mimics glutathione peroxidase.\textsuperscript{1} Another chemical modification methodology was introduced by Kaiser,\textsuperscript{2} who transformed the metallo-hydrolase carboxypeptidase A into an oxidase by substitution of the zinc (II) ion by copper-(II) (Figure 7.1). The oxidation of vitamin C was catalysed by Cu-carboxypeptidase A. Based on the results of Kaiser, we reasoned that substitution of the zinc (II) ion in the active site of the readily available metalloprotease, thermolysin, by a redox metal could transform the catalyst from a hydrolase into a peroxidase.

![Chemical structure](image)

Figure 7.1: Oxidation of vitamin C catalysed by metallo-substituted carboxypeptidase A

The essential zinc atom of thermolysin is likely involved either in substrate binding or catalysis, or both.\textsuperscript{3} A schematic presentation of the active site of thermolysin is shown in Figure 7.2. The Zn\textsuperscript{2+} atom is coordinated to two histidine residues (His-142 and His-146) and one glutamic acid (Glu-166).\textsuperscript{4} From the crystal structure (X-ray studies) it is known that the metal is tetrahedrally coordinated in the active site. Besides Zn\textsuperscript{2+} the protein contains four calcium atoms (not shown in Figure 7.2) which are responsible for enzyme stability.\textsuperscript{3,4}

![Schematic presentation](image)

Figure 7.2: Schematic presentation of the active site of thermolysin
Metal substitution of thermolysin and its application in oxygen transfer reactions

Metal substitution can be performed in three different ways: 1) direct replacement of the first metal by a second \(^5\), 2) biosynthesis of the metalloprotein under enriched conditions of the metal of choice \(^6\), and 3) removal of native metal by a metal binding agent and/ or competition with hydrogen ions at low pH, followed by insertion of the new metal. \(^7\) The third method is the easiest and the most applied method. By dialysis with a chelator, such as 1,10-phenanthroline (OP) or ethylenediamine-(N,N,N',N'')-tetra-acetic acid (EDTA) the metal atom can be removed and subsequently replaced by other metals. However, during the preparation of the apo-enzyme the protein structure could be changed irreversibly and therefore this method requires a stable apo-enzyme.

![Figure 7.3: Structure of the chelators OP (1) and EDTA (2)](image)

The mechanism of chelator action was described earlier. \(^7\) The stable ternary chelator (C) metal (M) enzyme (E) complex could be formed by a S\(_n\)1 (equation 1 and 2) or S\(_n\)2 (equation 3 and 4) type of reaction (Scheme 7.1). With EDTA, a S\(_n\)1 type of reaction takes place in which the rate-determining step is the dissociation of the enzyme metal ion complex (1). In this case the rate of inactivation is independent of chelator concentration. 1,10-Phenanthroline reacts by a S\(_n\)2 type of reaction resulting in the formation of a ternary enzyme-metal-chelator complex (3). This is followed by the dissociation of the chelator bound metal ion-enzyme complex. In this case the rate constant for OP is \(k_T\); the breakdown of the ternary complex into apo-enzyme E and CM\(^{n+}\).

\[
\begin{align*}
EM^{n+} & \rightleftharpoons K_d \quad E + M^{n+} \quad (1) \\
M^{n+} + C & \rightleftharpoons K_1 \quad CM^{n+} \quad (2) \\
EM^{n+} + C & \rightleftharpoons K_{EMC} \quad ECM^{n+} \quad (3) \\
ECM^{n+} & \rightarrow k_T \quad E + CM^{n+} \quad (4)
\end{align*}
\]

Scheme 7.1: Equations of the S\(_n\)1 (1 and 2) and S\(_n\)2 (3 and 4) reactions
Chapter 7

Metal substitution in thermolysin was earlier reported by Holmquist and Vallee. Crystals of the native enzyme were prepared and the zinc atom was removed by coordination with 1,10-phenanthroline (1) (Figure 7.3). They reconstituted the apo-enzyme with Zn$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and Cu$^{2+}$ to restore its catalytic activity.

To facilitate its handling and increase enzyme stability we investigated the isomorphous metal-substitution of immobilised thermolysin. We used two different kinds of chelators OP (1) and EDTA (2) to obtain the apo-enzyme. The reconstitution of the catalyst was performed with Zn$^{2+}$, Co$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, MoO$_4^{2-}$, SeO$_4^{2-}$, VO$_3^{2-}$ and WO$_4^{2-}$. The latter four ions have a more or less similar tetrahedral structure and were introduced in order to induce redox activity. Finally the reconstituted thermolysin was tested in oxygen transfer reactions with hydrogen peroxide as a clean oxidant.

Results and discussion

Metal substitution in the native enzyme

The apo-enzyme of thermolysin was prepared by removing the native zinc atom with a chelator by dialysis at optimal pH and temperature for the stability of the catalyst. Unfortunately, when OP was used in the preparation of the apo-enzyme on a large scale, more than 7 days were necessary to remove the zinc. For EDTA only 3 days were necessary, which was more favourable for the stability of the apo-enzyme (see Table 7.1).

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Ca$^{2+}$ (Log K$_i$)</th>
<th>Zn$^{2+}$ (Log K$_i$)</th>
<th>Activity (U/mg)</th>
<th>Time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (2 mM)</td>
<td>10.7</td>
<td>16.4</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>OP (2 mM)</td>
<td>1.1</td>
<td>6.4</td>
<td>0.02</td>
<td>7</td>
</tr>
</tbody>
</table>

The concentration of OP was probably too low to remove the Zn$^{2+}$ from the catalytic centre properly in a reasonable time period. According to the Bradford assay the amount of protein is 63 mg per gram of solid which indicates that the amount of adjuvants (stabilisers e.g. salts, other proteins and surfactants) is more than 90% of the total weight. The salts of the adjuvants could bind to OP and influence the binding rates (K$_{exc}$) in the formation of ECM$^{n+}$ complex (see scheme 7.1 reaction (3)). Besides, as shown in Table 7.1, the binding constant of EDTA towards zinc is much higher than that of OP.

The apo-enzyme was reconstituted with Zn$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and Cu$^{2+}$ as well as MoO$_4^{2-}$, SeO$_4^{2-}$, VO$_3^{2-}$ and WO$_4^{2-}$ to restore its catalytic activity (Table 7.2). To complete the substitution...
Metal substitution of thermolysin and its application in oxygen transfer reactions

A small amount of calcium chloride was added to maintain or restore enzyme stability. The activity of the enzyme preparations was assayed using FAGIA (3-(2-furylacyloyl)-glycyl-L-leucine) as chromogenic test substrate and compared with the results of Holmquist and Vallee.⁸

<table>
<thead>
<tr>
<th>Metals</th>
<th>Experimental</th>
<th>Literature⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (U/mg)</td>
<td>Restored activity (%)</td>
</tr>
<tr>
<td>Native enzyme</td>
<td>2800</td>
<td>-</td>
</tr>
<tr>
<td>Apo-enzyme</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Zn (II)</td>
<td>1.2</td>
<td>&lt;&lt;1</td>
</tr>
<tr>
<td>Co (II)</td>
<td>90</td>
<td>3.2</td>
</tr>
<tr>
<td>Cu (II)</td>
<td>21</td>
<td>0.8</td>
</tr>
<tr>
<td>Mn (II)</td>
<td>65</td>
<td>2.3</td>
</tr>
<tr>
<td>Mo (VI)</td>
<td>64</td>
<td>2.3</td>
</tr>
<tr>
<td>Se (VI)</td>
<td>40</td>
<td>1.4</td>
</tr>
<tr>
<td>V (V)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W (VI)</td>
<td>38</td>
<td>1.4</td>
</tr>
</tbody>
</table>

a: thermolysin was crystallised before metal substitution and the restored activity was calculated from the activity of highly purified enzymes

We can suggest several possible reasons for the much lower activity of the reconstituted enzyme observed by us compared to those obtained by Holmquist and Vallee⁸ (see Table 7.2). First, autolysis could occur, since it is known to take place when thermolysin loses one of its four calcium atoms.⁹,¹⁰ Holmquist and Vallee used OP as a chelator to remove the zinc ion. As shown in Table 7.1, the binding constant of this chelator to Ca²⁺ is ten orders of magnitude lower than that of EDTA, which reduces the effect of autolysis. The second reason lies in the method of calculation of the restored activities (U_out/ U_in). Our U_in corresponds to the activity of the (crude) enzyme before dialysis whereas Holmquist and Vallee first purified thermolysin by crystallisation and then performed the metal substitution which results in a lower enzyme activity and U_in value (see also Chapter 6; during crystallisation a decrease in activity of 20% could be expected). A third reason could be the stabilising effect of the tertiary crystal structure of the protein. It is known from the literature that enzyme crystals are more stable than native ones.¹¹ Finally, for the zinc-ion reconstitution it is known that an excess of zinc ions could bind to a second histidine group (His-231; see Figure 7.2) in the active site which is essential for its hydrolytic activity.⁴,⁹ This presumably occurred in our case, resulting in an insignificantly restored activity.
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Cobalt, copper and manganese were also used in excess to reconstitute the apo-thermolysin, but only a small fraction of the original activity was recovered. According to the literature the restored activity of Co\(^{2+}\)-thermolysin was twice as high as the native enzyme, which indicates that cobalt has a higher proteolytic potential than zinc in the active site of thermolysin. This behaviour was also observed by other groups\(^{12,13}\), which could be explained by the fact that cobalt requires a five coordinated geometry in the transition state.\(^{8,9,14}\) Co\(^{2+}\) can readily adjust from four into five coordination as it is thought to happen during catalysis and therefore, it is superior to Zn\(^{2+}\).

As shown in Table 7.2 some activity was recovered by reconstitution with molybdate, selenate and tungstate salts, whereas vanadate had no effect. We note that aqueous solutions of these salts did not show any hydrolytic activity towards PAGLA. Hence it is likely that these oxoanions are bond in the active site.

**Metal substitution in immobilised thermolysin**

To suppress the deactivation that we experienced with the free enzyme, we repeated the metal exchange experiments with thermolysin CLECS and with enzyme attached to Eupergit C (see Table 7.3). To obtain apo-enzyme from the CLECS or polymer bound thermolysin, the solid catalyst was washed several times with EDTA solutions followed by centrifugation or filtration. This treatment was repeated until the residual activity was lower than 1% of the original hydrolytic activity. When OP was used, the apo-catalyst still contained 10% residual activity after one week washing whereas for EDTA the metal was removed within three days. Apparently immobilised thermolysin is more stable and requires a strong chelator to remove the zinc ion from the active site.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Native enzyme (2800 U/mg)</th>
<th>CLECS (347 U/mg)</th>
<th>Fixed to Eupergit C (12 U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (U/mg)</td>
<td>Restored activity (%)</td>
<td>Activity (U/mg)</td>
</tr>
<tr>
<td>Zn (II)</td>
<td>1.2</td>
<td>&lt;&lt;1</td>
<td>23</td>
</tr>
<tr>
<td>Co (II)</td>
<td>90</td>
<td>3.2</td>
<td>66</td>
</tr>
<tr>
<td>Cu (II)</td>
<td>21</td>
<td>0.8</td>
<td>19</td>
</tr>
<tr>
<td>Mn (II)</td>
<td>65</td>
<td>2.3</td>
<td>65</td>
</tr>
<tr>
<td>Mo (VI)</td>
<td>64</td>
<td>2.3</td>
<td>0</td>
</tr>
<tr>
<td>Se (VI)</td>
<td>40</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>W (VI)</td>
<td>38</td>
<td>1.4</td>
<td>0</td>
</tr>
</tbody>
</table>

\(a\): the restored activity was calculated from the native immobilised enzyme preparations.
Metal substitution of thermolysin and its application in oxygen transfer reactions

Reconstitution of the immobilised apo-enzyme with different metal ions and, subsequently, calcium resulted in a modest recovery of activity. Compared to the free enzyme the loss of activity was much reduced for the CLECs although the restored activity was still very low (7 U/mg). This could be explained by the auto-digestive behaviour of the enzyme crystals. The crystals are in close proximity and tightly bonded and the loss of a calcium atom during the formation of apo-CLECs could create an enzyme molecule that is auto-digestive towards its neighbours. We obtained the highest hydrolytic activity with cobalt-substituted thermolysin CLEC (66 U/mg with a restored activity of 19%). A closer look under the microscope showed that the crystal structure of the catalyst was still intact, suggesting isomorphous metallo-substitution. However, earlier experiments of most metals used for reconstitution of the apo-enzymes showed that protein refolding occurred.\textsuperscript{10,14-16} To substantiate the isomorphous substitution in our case X-ray studies of the crystals should be made.

The restored activities of metallo-substituted thermolysin on Eupergit C were, with the exception of Cu (II)-ion, greater than 50%. Even with bulky ions, such as molybdate (VI), selenate (VI) or tungstate (VI), it was possible to reconstitute the Eupergit C-immobilised apo-enzyme. We note that under similar conditions the reconstitution of the apo-CLEC failed. It would seem that the CLECs are too restricted conformationally to accept bulky ions or less cationic groups were available (due to crosslinking with glutaraldehyde) to bind with the oxo-anions.

Application of metal-substituted thermolysin in oxygen transfer reactions

Tungsten is a well-known oxidation catalyst.\textsuperscript{17-19} In order to check the change of thermolysin’s functionality from hydrolysis to oxidations we used tungstate-thermolysin to catalyse sulfoxidation reactions (Figure 7.4). The tungstate-substituted immobilised thermolysin on Eupergit C with a proteolytic activity of 7 U/mg was used for the conversion of a number of sulfides. The oxidation was performed with 2 equivalents H\textsubscript{2}O\textsubscript{2} titrated in 4 hours at 40°C in tert-butyl alcohol. Whereas most enzymes lose catalytic activity under these reaction conditions\textsuperscript{20} our catalyst showed high stability towards solvent, oxidant and temperature.

\[
\begin{align*}
\text{R}_1\text{S}^\cdot &\text{O} \quad \text{H}_2\text{O}_2 \quad \text{R}_1\text{S}^\cdot \text{O} \quad \text{H}_2\text{O}_2 \\
\text{R}_1 &\quad \text{R}_2 \\
(3) &\quad (4) \\
\text{O} &\quad \text{S}^\cdot \text{O} \\
\text{R}_1 &\quad \text{R}_2
\end{align*}
\]

Figure 7.4: Enzymatic catalysed sulfoxidation reactions

The redox properties of tungstate-thermolysin were investigated in the oxidation of thioanisole by hydrogen peroxide. After 5 hours (70% conversion) the reaction rate declined
because nearly all sulfide (3) had been consumed. The sulfoxide (4) was subsequently converted into sulfone (5) in a slow reaction that took 24 hours to reach complete conversion.

In the absence of any catalyst the reaction was much slower and progressed linearly over time to reach 48% conversion to 4 in 7 hours. No further reaction to the sulfone was observed. When sodium tungstate was used as catalyst 47% conversion was observed. We also performed a reaction in the presence of tungstate thermolysin and removed the catalyst by filtration after 2.5 hours, when the maximum reaction rate had been achieved (at 9% conversion). The reaction slowly continued in the filtrate at a rate comparable to that of the uncatalysed reaction, to reach 55% conversion of 4 after 7 hours.

It is known from the literature that thermolysin prefers substrates with bulky, hydrophobic substituents. Accordingly we assessed tungstate-thermolysin in the oxidation of a number of aromatic sulfides (Table 7.4) under similar conditions as used in the oxidation of thioanisole.

Table 7.4: Oxidation of sulfides catalysed by W-thermolysin on Eupergit C

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>Time (h)</th>
<th>Yield(^a) (%)</th>
<th>Background yield(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sulfoxide</td>
<td>Sulfone</td>
</tr>
<tr>
<td>(6) (\text{Ph}S^-)</td>
<td>7</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>(7) (\text{PhSC}_6\text{H}_5)</td>
<td>24</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>(8) (\text{PhS}_2\text{C}_6\text{H}_5)</td>
<td>24</td>
<td>86</td>
<td>11</td>
</tr>
<tr>
<td>(9) (\text{PhS}O\text{C}_6\text{H}_5)</td>
<td>2</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>(10) (\text{PhS}_2\text{NH})</td>
<td>20</td>
<td>82</td>
<td>18</td>
</tr>
</tbody>
</table>

\(^a\) the yields were determined by HPLC

The conversion of diphenyl sulfide (7) in the presence of tungstate-thermolysin was insignificant (1%) compared to the background reaction. The lack of activity is probably caused by substrate selectivity because phenyl benzyl sulfide (8), containing one carbon atom extra, gave a conversion to 86% sulfoxide and 11% sulfone within 24 hours.
Metal substitution of thermolysin and its application in oxygen transfer reactions

The highest reaction rate was observed with phenyl mercaptoacetophenone (9). Within 2 hours complete conversion of 9 was observed, giving a mixture of sulfoxide and sulfone (Figure 7.5). After 5 hours complete conversion to the sulfone was observed. In the absence of any catalyst, or in the presence of Na₂WO₄, apo- or native thermolysin approximately a 35% conversion to the sulfoxide was observed after 24 hours. No formation of sulfone took place under these conditions. We tentatively attribute the much higher reactivity of 9 compared to 8 with its closer resemblance to the natural substrates (peptides) of thermolysin. Hence, this result supports the hypothesis that tungstate is incorporated in the active site.

![Figure 7.5: Tungstate-thermolysin catalysed oxidation of phenyl mercaptoacetophenon (9); (o) sulfide, (□) sulfoxide (△) sulfone](image)

Phenothiazine 10 (see Table 7.4) has a rigid, planar structure compared to 7 and 8 but reacted faster. After 20 hours a complete conversion of the sulfide into the sulfoxide was reached and already 18% sulfoxide was converted into sulfone. It seems that the acceptance of the bulky substrates by the active site is very high compared to most peroxidases²² which could be explained by the flexibility of the enzyme. However, for all catalysed oxygen transfer reactions no enantioselectivity was obtained for the sulfoxides.

Conclusions

The reconstitution of free apo-thermolysin with a range of metal and oxometal ions resulted in a low recovery of the proteolytic activity. This is ascribed to autodigestion in the course of the reconstitution. The best reconstitution yield was obtained with thermolysin attached to Eupergit C.

Tungstate thermolysin is a moderately active catalyst for the oxidation of sulfides into their sulfoxides and sulfones. It accepts much bulkier reactants than most peroxidases, but the sulfoxides are racemic. Nevertheless, by changing the metal in the active site the functional properties of thermolysin were changed from hydrolytic- towards peroxidase activity.
Chapter 7

Experimental procedure

Materials

Thermolysin from *Bacillus thermoproteolyticus rokko* (E.C. 3.4.24.4) was a gift from DSM, Eupertig C 250 L was a gift from Röhm GmbH (Darmstadt, Germany). 3-(2-Furylacryloyl)-glycyl-L-leucine amide was purchased from Sigma, CLECS from Altus Biologics Inc., hydrogen peroxide (35 %) from Merck and Na$_2$SO$_3$, Na$_2$SO$_4$ from J. T. Baker. The sulfides thioanisole, diphenyl sulfide, diphenyl sulfoxide, phenyl benzyl sulfide, phenothiazine and oxindole, Na$_2$MoO$_4$, Na$_2$SeO$_4$, VO$_3$, Na$_2$WO$_4$, MnAc$_2$, CoCl$_2$, ZnAc$_2$, CuCl$_2$ were obtained from Aldrich. 1,3-Dimethoxybenzene, 1,2,3-trimethoxybenzene, thiophenol and 2-bromoacetophenone, 1,10-phenanthroline (OP) and ethylenediamine-(N,N,N',N')-teta-acetic acid (EDTA) were purchased from Acros.

Analysis and equipment

Thermolysin was dialysed in a Stirred Ultrafiltration Cell (Amicon model 8050) with a Diaflow Ultrafiltration membrane 10 kDa (YM regenerated cellulose) under nitrogen gas pressure of 75 psi. UV measurements, to follow the hydrolysis of FAGLA, were performed on a Cary 3 spectrophotometer from Varian. The data points from hydrolysis of FAGLA were transformed to DOS and fitted with RR-graph (see also Chapter 6).

The sulfoxidation reactions were monitored on a reversed phase HPLC on a custom-packed Symmetry C$_{18}$ cartridge (Waters Radial-Pak, 8 x 100 mm, 7 μm) contained in a Waters RCM 8x10 compression unit, with a Waters 486 tunable absorbance detector with Waters Millenium$^{32}$ Software. The products of thioanisole oxidation were analysed using 1,2,3-trimethoxybenzene as internal standard and acetonitrile/water 35:65 (v:v) as eluent (flow 1 ml min$^{-1}$), with detection at 220 nm. At similar wavelength the products of diphenyl sulfide oxidation were analysed using 1,3-dimethoxybenzene as internal standard and acetonitrile/water 40:60 (v:v) as eluent. The reaction with benzyl phenyl sulfide were analysed using 1,2,3-trimethoxybenzene as internal standard and acetonitrile/water 45:55 (v:v) as eluent. For phenyl mercaptoacetophenone oxidation the components were analysed using acetonitrile/ water (40:60 (v:v)) as eluent (flow 1.0), with detection at 220 nm and oxindole as internal standard.

Samples for analysing enantioselectivity of the sulfoxides were diluted with a hexane/isopropyl alcohol mixture of 75:25 (v:v) and dried over Na$_2$SO$_4$. After centrifugation, the samples were injected on chiral HPLC using a Chiralcel OD column (Diacel Chemical Industries, Ltd., 250 X 4.6 mm), eluent (flow 0.6 ml min$^{-1}$) hexane/isopropyl alcohol 75:25 (v:v) and detected on a Waters 486 tunable absorbance detector at 220 nm with Waters Millenium$^{32}$ software. A Metrohm Dosimat 665 was used for addition of reagents and metal solutions. All reactions were performed in a 50 ml thermostat controlled temperature reaction vessel.
Metal substitution of thermolysin and its application in oxygen transfer reactions

**Metal substitution of thermolysin**

The amount of zinc in buffers, salts and other reagents was minimised by using only reagent grades and extracting the distilled water with dithizone/carbon tetrachloride as presented by others.\(^{22}\) It was possible to obtain solutions containing very low amounts of metal ions. The buffers were prepared with metal free water and adjusted at optimal pH.

By dialysis of 1 g of thermolysin with 3 L of EDTA solution (2 mM EDTA, 50 mM TRIS, pH 7.5) at 4°C the apo-enzyme was achieved within three days. Under similar conditions OP was used except a longer time period and more washing solvent was necessary to remove the metal from the active site. The apo-enzyme was washed with 500 ml buffer (1 M NaCl, 50 mM TRIS, pH 7.5) during a 72 hours. The reconstitution of the enzyme was performed by adding to a 100 μl apo-enzyme solution at 4°C stepwise, by a dosimat 665, 10 μl of 1 mM metal solution (in 1 M NaCl, 50 mM TRIS, pH 7.5) during one hour. After every 10 minutes 10 μl was removed from the mixture and measured for activity. Finally a small amount of CaCl₂ was added to each sample and the mixture was shaken for 16 hours at 4°C. The reconstituted thermolysin was measured for its hydrolytic activity according to the FAGLA assay. In the case of substitution with zinc, 200 μl of apo enzyme was used and the zinc solution (10 ml 0.01 mM) was titrated during 8 hours with a dosimat at 4°C. Samples were taken and CaCl₂ was added immediately. After 16 hours of shaking the activity was measured.

Thermolysin was immobilised on Eupergit C as described in Chapter 6. For metal substitution of immobilised thermolysin, 1 g of catalyst was shaken in 100 ml 2 mM EDTA solution (1M NaCl, 50 mM TRIS buffer, pH 7.5) at 4°C. After 3 hours the solids were collected by centrifugation (CLECS) or filtration (thermolysin on Eupergit C) and a new solvent with chelator was used. Finally after three days when the catalyst contained less than 1 % of its original hydrolytic activity, the excess of EDTA was removed by washing the solids during one day with brine (3 x 200 ml 1M NaCl, 50 mM TRIS, pH 7.5). The reconstitution of the apo-enzyme was performed by adding a 1 mM of metal solution (in 50 mM TRIS pH 7.5) to the catalyst and mixing for 1 hour at room temperature. The volume was measured and the Ca\(^{2+}\) concentration was increased to 10 mM. After shaking for one night at 4°C the activity of the metal-substituted catalyst was measured. The reconstitution with zinc was performed by the titration of a diluted zinc solution (0.1 mM ZnAc, 50 mM TRIS) to the apo-enzyme at 4°C in 6 hours.

**Activity measurements**

The hydrolysis of FAGLA, 3-(2-furylacryloyl)-glycyl-L-leucine amide, was measured according to the method developed by Feder\(^{23}\) improved by our own experience (see Chapter 6). In 100 ml TRIS buffer (50 mM pH 7.5, 10 mM CaCl₂) 76.6 mg of substrate (2.49 mM) was dissolved. In a quartz cuvet 3 ml of substrate were mixed with 1-5 mg of catalyst (in case of immobilised thermolysin on Eupergit C 5-10 mg) for 3 seconds and immediately placed in the
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UV-spectrophotometer. During stirring the decrease of absorbance was measured at 345 nm. After fitting the data points to the curve with the function \( [S] = k_2 \cdot e^{-kt} \) the activity in U/mg catalyst could be calculated (see Appendix of Chapter 6). Units (U) were defined in \( \mu \text{mol FAGLA min}^{-1} \) per mg of enzyme.

**Synthesis of substrates and products**

Phenyl mercaptoacetophenone (9) was prepared by adding 5 g of 2-bromoacetophenone (25 mmol) to 50 ml cold ethanol. After 6.9 g \( \text{K}_2\text{CO}_3 \) (50 mmol) was added, 1 equivalent of thiophenol (25 mmol) was slowly titrated during 2 hours. The reaction was stirred for 12 hours and the solid was filtrated off. Water was added to remove the excess of potassium bromide from the mixture. The combined aqueous layer was extracted with ethyl acetate. The organic layer was evaporated and the obtained yellow crystals were recrystallised with ethyl acetate/ether mixture. Yield 83 %, purity (HPLC) 99%. Mp: 227-229°C. \(^1\)H NMR (400 MHz, CDCl\(_3\)) 4.25 (s, 2H), 7.25-8.22 (m, 10H). MS: m/e = 228 (C\(_9\)H\(_{12}\)SO).

The chemical sulfoxidations were prepared in different ways to obtain sulfoxide 4 and sulfone 5. The sulfoxide was prepared according to the literature\(^{23}\), by dissolving 0.2 g sulfide (1.6 mmol) in 4 ml acetic acid. After cooling the mixture down to -20°C one equivalent of concentrated \( \text{H}_2\text{O}_2 \) (9 M) was added. The reaction mixture was stirred for 12 hours and the acetic acid was removed by evaporation. The solid was diluted with 10 ml H\(_2\)O saturated with Na\(_2\)SO\(_4\). The aqueous phase was extracted with ether and finally the organic layer was dried over Na\(_2\)SO\(_4\) and evaporated to obtain the sulfoxide 4 (1.15 mmol, 72%).

The sulfone 5 was prepared according Trost et al.\(^{25}\), by dissolving 1.5 g of sulfide (12 mmol) in 40 ml methanol at 0°C. Three equivalents of KHSO\(_3\) (36 mmol) solubilised in 40 ml H\(_2\)O was added. The temperature was raised to room temperature during 4 hours and the mixture was diluted with water. The organic layer was extracted with chloroform (3x) and the combined organic phase was washed with water end dried over Na\(_2\)SO\(_4\). The liquid was evaporated and the sulfone 5 was afforded (10 mmol, 83%).

**Standard oxidation procedure**

In the standard procedure substrate (0.8 mmol) and wet immobilised tungstate-thermolysin (200 mg) were mixed in tert-butyl alcohol and stirred at 40°C in a sealed vessel. After the substrate was solubilised two equivalents (1.6 mmol) of the diluted oxidant (1M H\(_2\)O\(_2\)) were titrated with a Metrohm 665 dosimat during 2 hours to the reaction vessel. Samples were taken after 15 minutes and mixed with Na\(_2\)SO\(_3\) to destroy the oxidants. In the case of phenyl mercaptoacetophenone catalase was used. The course of the reaction was monitored by HPLC.
Metal substitution of thermolysin and its application in oxygen transfer reactions

References
Chapter 7


Summary

Immobilisation of metallo-enzymes and their application in non-natural conversions

This thesis deals with various aspects involved in the application of metallo-hydrolases as catalysts in organic synthesis. Aminoacylase and thermolysin were chosen because they are readily available industrial catalysts. Furthermore, detailed studies on their catalytic activity in aqueous solvents and their stabilities in organic media have been published, providing a basis to investigate their behaviour, applications and stability in organic synthesis.

Aminoacylase and thermolysin belong to the family of the proteases in which they represent the subclasses of the exopeptidases (aminoacylases) and endopeptidases (thermolysin). Both proteases contain a metal ion Zn$^{2+}$ in the active site, which is essential for its catalytic activity. The relationship between the two enzymes and their hydrolytic mechanism is discussed in Chapter 1. An introduction to the immobilisation, colyphilisation and modification of enzymes, which are used to increase stability, operational life and solubility in organic solvent, is presented. As regards industrial applications, each of these methods has advantages and disadvantages, which should be considered in relation to the specific application.

Chapter 2 presents the immobilisation of aminoacylase, chloroperoxidase and phytase into polyurethane foams. These enzymes are heavily glycosylated (upto 46% of their total mass) and this abundance of reactive groups makes them eminently suitable for covalent binding into PUR-foams. With aminoacylase a very high enzyme loading of 187 mg g$^{-1}$ was obtained with an immobilisation efficiency of 60%. The immobilisation yield of 100% and the operational stability during many cycles without any protein leakage makes the method suitable for industrial
Summary: Immobilisation of metallo-enzymes and their application in non-natural conversions

applications. Hence, we conclude that the immobilisation method is widely applicable to glycosylated enzymes.

In Chapter 3 the results of different immobilisation methods applied to aminoacylase are described. Immobilisation of aminoacylase on DEAE-Sephadex, which is used in the commercial hydrolysis of N-acetyl-D,L-methionine, was compared to other immobilisation methods, such as covalent immobilisation into polyurethane foams and immobilisation on DEAE-cellulose.

We investigated the use of immobilised aminoacylase as a transesterification catalyst in hexane. The native enzyme was stable and readily mediated the acylation of secondary alcohols with absolute enantioselectivity. Unfortunately the catalytic activity of the immobilisates under the same conditions was low. Apparently, the immobilisation techniques that have been used do not protect the enzyme against loss of water. Hence, the choice of the immobilisation method depends on the specific application of the catalyst and involves a trial and error approach as shown by the results in the hydrolysis and transesterifications reactions. Aminoacylase PUR-foams have a higher catalytic activity in aqueous medium compared to covalently bonded enzyme on Eupergit C whereas in organic solvent the reverse is true.

The native aminoacylase has been employed in the transesterification of a wide range of substrates as described in Chapter 4. The selectivity in the transesterification of 1-phenylethanol was investigated using a range of acyl donors with different chain lengths. Optimum activity was observed with vinyl butyrate. The enzyme mediated the acylation of a wide range of chiral secondary alcohols with essentially absolute stereospecificity (E>500). Large hydrophobic groups of the alcohols were accepted in the active site, which is a distinct advantage compared with the lipases that commonly have been used in similar reactions.

Chapter 5 deals with the superactivation of aminoacylase in organic media by colyophilisation with surfactants. Increased space-time yields up to 2 kg L⁻¹ d⁻¹ and a productivity 127 g (g⁻¹ protein) h⁻¹ were obtained in the transesterification of 1-phenylethanol in a reaction medium with substrates and products as solvent. A remarkable difference in efficiency was observed for the aminoacylase catalysed transesterification: the native aminoacylase from Aspergillus melleus was more active than the one from Aspergillus oryzae whereas the reverse was true for the aerosol-coated enzyme. We note that the type of surfactant, the amount, the pH and the salt concentration influence the residual enzyme activity. The high activity of the aminoacylase colyophilisates in transesterification and their acceptance of a wide scope of substrates (as shown in Chapter 4) makes them eminently suited for industrial applications.

The hydrolytic activity of thermolysin was measured by two different methods, the digestion of casein and the hydrolysis of FAGLA, as described in Chapter 6. The hydrolysis of the latter substrate was described according to pseudo-first order reaction kinetic and was favourable compared to the casein hydrolysis. The casein assay was less sensitive and the detection was less reliable.
Summary: Immobilisation of metallo-enzymes and their application in non-natural conversions

Thermolysin was immobilised as cross-linked enzyme crystals, as well as covalently bonded to Eupergit C. Attempts to use the immobilised protease in ammonolysis failed. However, increased stabilities were obtained in organic media which made the immobilisates suitable for metal-substitution as described in Chapter 7.

Metal-substitution of native thermolysin was not successful, but with immobilised thermolysin a large fraction of the original activity (up to 85%) was restored. The reconstitution of apo-thermolysin immobilised on Eupergit C succeeded with metal ions such as molybdate, selenate and tungstate, whereas the CLECs had some restrictions in this respect.

Tungstate-thermolysin was tested as a catalyst for the oxidation of thioethers to sulfoxides using H₂O₂ as the oxidant. Despite the absence of enantioselectivity the discrimination of certain substrates compared with the background reaction suggests that the metal was correctly bound in the active site. Furthermore, the fundamental properties of thermolysin are changed from hydrolysis towards oxidation. The method creates more possibilities in the field of modification of enzymes to increase its application.

According to their nature aminoacylase and thermolysin were built to perform hydrolysis, but in organic medium they can perform synthesis. As shown in this thesis for both catalysts as readily available enzymes, their possibilities are much wider in biotransformations.
Samenvatting

Immobilisatie van metaalenzymen en de toepassing ervan in organisch medium

In dit proefschrift zijn verschillende aspecten beschreven van de toepassing van aminoaclase en thermolysine in de organische synthese. Beide enzymen zijn gekozen omdat het stabiele en goed verkrijgbare industriële enzymen betreft. Er is veel bekend in de literatuur over de katalytische activiteit van de enzymen en hun stabiliteit in organisch milieu. Dit maakt aminoaclase en thermolysine uitermate geschikt om hun gedrag, toepassing en stabiliteit in het organisch medium te onderzoeken.

Aminoaclase en thermolysin behoren tot de familie van de proteases. Deze groep kan gesplitst worden in twee subgroepen: exo-peptidasen (aminoaclase) en endo-peptidasen (thermolysine). Beide enzymen bevatten een zink atoom in de active site welk essentieel is voor de katalytische activiteit. De overeenkomsten en verschillen tussen aminoaclase en thermolysine als metaalenzymen en hun reactiemechanismen zijn weergegeven in Hoofdstuk 1. Ook wordt er een beschrijving gegeven over mogelijkheden om enzymen stabiler, meer levensvatbaar en beter oplosbaar te maken in organisch milieu, zoals immobilisatie, colyphilisatie en modificatie. De drie methoden hebben elk hun voor- en nadelen met betrekking tot de katalytische activiteit en enantioselectiviteit van het enzym. De verschillende technieken moeten tegen elkaar worden afgewogen in relatie tot de specifieke probleemstelling.

In Hoofdstuk 2 is de immobilisatie van aminoaclase, chloorperoxidase en fytase in polyurethaanschuim beschreven. Via een covalente binding zijn de enzymen gekoppeld aan het polymer. Mede doordat de drie enzymen een groot aantal reactieve suikergroepen bevatten aan het eiwitoppervlak zijn ze uitermate geschikt om een covalente binding aan te gaan met het
dragemateriaal. Met aminoacylase is een zeer hoge belading bereikt van 187 mg eiwit per gram schuim met een efficiency van 60% en een immobilisatie opbrengst van 100%. De activiteit van aminoacylase-PUR blijft hoog als het meerdere keren achter elkaar gebruikt wordt. Onder de reactiecondities laat er geen enzyme los van de drager. Mede hierdoor kan de methode geschikt zijn voor industriële doeleinden. Een belangrijke conclusie is dat deze immobilisatiemethode geschikt is voor alle geglycosyleerde enzymen.

Aminoacylase is via verschillende methode geïmmobiliseerd, de resultaten hiervan zijn beschreven in Hoofdstuk 3. De immobilisatie van aminoacylase op DEAE-Sephadex, die gebruikt wordt in de industriële hydrolyse van N-acetyl-D,L-methionine, is vergeleken met andere immobilisatie methoden, zoals covalente immobilisatie op polyurethaan schuim en immobilisatie op DEAE-cellulose. De geïmmobiliseerde preparaten zijn uiteindelijk getest in de oomestering van 1-phenylethanol in hexaan. Het natieve enzym is stabiel en katalytisch actief in de aclylering van secundaire alcoholen met een hoge enantioselectiviteit (E>500). Het geïmmobiliseerde enzym is minder actief onder gelijke omstandigheden. Welke immobilisatie methode geschikt is voor aminoacylase, hangt af van de specifieke toepassing. Immers, door immobilisatie kunnen specifieke eigenschappen van het enzym veranderen, waardoor de activiteit en selectiviteit veranderd kan worden in organisch milieu. Zo zijn bijvoorbeeld de aminoacylase PUR-schuimen actiever in hydrolyseracties dan aminoacylase geïmmobiliseerd op Eupergit C, in hexaan medium is dit echter omgekeerd.

In Hoofdstuk 4 worden de resultaten gepresenteerd van de aminoacylase gekatalyseerde aclylering van chirale alcoholen. Diverse acyldonoren werden getest in de transverstering van 1-phenylethanol. De hoogste reactiesnelheid werd verkregen met vinyl butyraat. Daarnaast is vastgesteld dat aminoacylase enantioselectief is voor een grote groep aromatische secundaire alcoholen. Het katalytisch centrum accepteert vele hydrofobe alcoholen; dit is een voordeel ten opzichte van de lipasen die meestal worden gebruikt voor de resolutie van alcoholen.

Van aminoacylase is een superactieve katalysator gemaakt door het te colyophiliseren met surfactants. Een transverstering met een space-time yield van 2 kg per liter per dag en een productiviteit van 127 g per g eiwit per uur is beschreven in Hoofdstuk 5. De transverstering kan zonder oplosmiddel worden uitgevoerd met zeer hoge opbrengsten.

De aminoacylases afkomstig van de schimmels Aspergillus melleus en Aspergillus oryzae verschillen qua katalytische activiteit. Het natieve aminoacylase geproduceerd door A. oryzae is minder actief in transesterificaties dan het enzym van A. melleus. Het AOT-conjugaat van aminoacylase van A. oryzae is echter veel actiever dan het overeenkomstige preparaat van de A. melleus. Verder is gebleken dat het type surfactant, de pH en de zoutconcentratie van belang kunnen zijn gedurende het colyophiliseren van enzymen. Onder optimale condities kan het gecolyophiliseerde enzym preparaat van aminoacylase uitermate geschikt zijn voor industriële
Samenvatting: Immobilisatie van metaalenzymen en de toepassing ervan in organisch medium

toepassingen, mede door de hoge enantioselectiviteit van het enzym voor vele aromatische secondaire alcoholen als substraat.

In Hoofdstuk 6 zijn de resultaten beschreven van de immobilisatie van thermolysine. Twee activiteitsmetingen zijn ontwikkeld en vergeleken met elkaar: de hydrolyse van caseïne en FAGLA. De laatstgenoemde kan met een pseudo-eerste orde reactie kinetiek beschreven worden.

Thermolysine is op twee manieren geïmmobiliseerd: als crosslinked enzym crystals (CLECS) en via covalente multipoint attachment op Eupergit C. Om de stabiliteit van het geïmmobiliseerde enzym preparaat te testen is het gebruikt in een ammonolyse reactie met tert-butylalcohol als oplosmiddel. Beide enzymepreparaten bleken niet of nauwelijks actief te zijn onder deze condities.

De verhoogde stabiliteit van geïmmobiliseerd thermolysine heeft voordelen bij metaalsubstitutie, zoals beschreven is in Hoofdstuk 7. Uiteindelijk is het gehukt om in thermolysine, geïmmobiliseerd op Eupergit C, het zink atoom te vervangen door molybdzaat, selenaat en wolframzaat. Het verlies van activiteit gedurende de substitutie is op deze manier geminimaliseerd. De CLECS blijken beperkingen te hebben in de metaalsubstitutie en accepteren de genoemde metalen niet.

De metaalgesubstitueerde enzymen zijn uiteindelijk getest op hun redoxactiviteit. Wolframzaat thermolysine op Eupergit katalyseerde de oxidatie van een aantal thioethers tot de overeen-komstige (racemische) sulfoxiden en sulfonen. De hoogste activiteit werd verkregen met phenyl-mercaptoacetophenon dat qua structuur overeenkomst vertoont met de natuurlijke reactant. Ondanks het onbreken van enantioselectiviteit suggereren de katalytische activiteit en selectiviteit van het metaalgesubstitueerde enzym dat het metaal op de juiste positie in het apo-enzyme terecht is gekomen. Hierdoor is het mogelijk gebleken dat thermolysin naast hydrolyse reacties ook oxidaties kan uitvoeren d.m.v. metaalsubstitutie.

De industriële enzymen, aminoaacylase en thermolysine, bezitten van nature een hoge enantioselectieve hydrolytische activiteit. Zoals in dit proefschrift is gebleken zijn ze eveneens actief in het organisch medium en hebben ze veel meer mogelijkheden als biokatalysator.
Dankwoord

Na dik vier jaren zweten, ploeteren en lachen is het gelukt om een boekwerk te produceren waarmee tevens een einde is gekomen aan mijn promotieonderzoek. Uiteraard heb ik dit resultaat niet alleen kunnen volbrengen en wil ik dan ook in dit gedeelte van mijn proefschrift gebruik maken om een aantal mensen te bedanken.

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List of publications

F. Van de Velde, N. D. Lourenço, M. Bakker, F. Van Rantwijk, R. A. Sheldon
Improved operational stability of peroxidases by coimmobilization with glucose oxidase.

F. Van de Velde, M. Bakker, F. Van Rantwijk, R. A. Sheldon
Chloroperoxidase-catalyzed enantioselective oxidation in predominantly organic media.

F. Van de Velde, M. Bakker, F. Van Rantwijk, G. P. Rai, L. P. Hager, R. A. Sheldon
Engineering chloroperoxidase for activity and stability.

M. Bakker, A.S. Spruijt, F. Van de Velde, F. Van Rantwijk, R.A. Sheldon
Enantioselective transesterification of secondary alcohols mediated by aminoacylases from *Aspergillus* species.

M. Bakker, F. Van Rantwijk, R. A. Sheldon
Aminoacylases for the acylation of alcohols and amines
*US patent application*, date 24-9-1999 (*nr. 99 118 844.2*)

M. Bakker, F. Van de Velde, F. Van Rantwijk, R. A. Sheldon
Highly efficient immobilization of glycosylated enzyme into polyurethane foams.

M. Bakker, A.S. Spruijt, F. Van Rantwijk, R. A. Sheldon
Highly enantioselective, amino-acylase catalyzed transesterification of secondary alcohols.

M. Bakker, F. Van Rantwijk, R. A. Sheldon
Efficient transesterifications mediated by surfactant-conjugates of aminoacylases.
Curriculum Vitae

Martin Bakker werd op 3 maart 1970 geboren te Den Burg. In 1989 behaalde hij zijn VWO diploma aan de OSG De Hoge Berg in Den Burg. In datzelfde jaar begon hij met zijn studie Scheikunde aan de Universiteit van Amsterdam. Het afstudeeronderzoek is uitgevoerd op de Bioorganische Vakgroep onder begeleiding van Prof. dr. U. K. Pandit, waar gewerkt is aan de synthese van een transition state analogon voor katalytische antilichamen. Vervolgens heeft de auteur een bedrijfsstage gevolgd op de afdeling Medische Chemie van Solvay Pharmaceuticals te Weesp, waar gewerkt is aan de ontwikkelingen van nieuwe methoden om biologische actieve stoffen voor dopamine en serotonine receptoren te synthetiseren. In 1995 werd het doctoraal examen Scheikunde behaald en aansluitend is er gestart met een promotieonderzoek aan de Technische Universiteit Delft. Onder supervisie van Prof. dr. R. A. Sheldon is er onderzoek gedaan naar het immobiliseren van metallo-proteases en de toepassing van deze enzymen in organisch milieu. Momenteel is de auteur werkzaam bij Avantium Technologies B.V. te Amsterdam.