Immobilization of Trypsin for Peptide Synthesis and Hydrolysis Reactions

zur Erlangung des akademischen Grades eines
DOKTORS DER INGENIEURWISSENSCHAFTEN (Dr.-Ing.)

der Fakultät für Chemieingenieurwesen und Verfahrenstechnik des
Karlsruher Instituts für Technologie (KIT)
vorgelegte

genehmigte
DISSERTATION

von
Dipl. Biol. (t.o.) Julia Andre geb. Stolarow
aus Odessa, Ukraine

Referent: Prof. Dr. rer. nat. Christoph Syldatk
Korreferent: Prof. Dr.-Ing. Rudolf Hausmann
Tag der mündlichen Prüfung: 19. November 2015

Für meine Eltern

Acknowledgements

I wish to express my sincere thanks to people who continuously supported and guided me throughout my work.

Prof. Dr. rer. nat. Christoph Syldatk for giving me the opportunity to conduct my research work at his group, his guidance and his enormous scientific knowledge giving me a great opportunity to learn.

Prof. Dr.-Ing. Rudolf Hausmann for his supervision, productive scientific discussions, his motivating personality and professional advice helping me to overcome many obstacles in my research work.

Former and current members of the Technical Biology group for the great friendly and supporting atmosphere during my work and spare time: Dr.-Ing. Ines Schulze, Dr. rer. nat. Mareike Perzborn, Laura Krämer, Dr.-Ing. Berna Gerçe, Dr.-Ing. Martin Pöhnlein, Dipl.-Ing. Melanie Gerlitzki, Dipl.-Biotechnol. Johannes Kügler, Dr.-Ing. Marius Henkel, Dr. rer. nat. Markus Andre, Dr.-Ing. Ulrike Engel, M. Sc. Janina Beuker, Dipl.-Ing. Michaela Zwick, M. Sc. Judit Willenbacher, M. Sc. Sarah Dold, M. Sc. Christin Slomka, Sandra Baumann, Dr.-Ing. Katrin Ochsenreither, Dipl.-Ing. Florian Oswald, Desiree Westermann, Werner Mandel, Harald Gotzmann, Siegfried Almstedt, Katja Rupp, Susanne Warth, M. Sc. Oliver Buß, Dipl. Biol. (t.o.) Sascha Siebenhaller, M. Sc. Stefan Dörsam.

All the students who contributed to the research in this work by constant diligence and commitment in laboratory: Dipl.-Ing. Johanna Stegmann, M. Sc. Natalja Beying, B. Sc. Leonie Schultz, Angelo Dalli, Dipl.-Ing. Steffen Hildebrandt, B. Sc. Benjamin Voß, B. Sc. Manuel Heinzelmann, M. Sc. Wladimir Yeremchuk, B. Sc. Simon Reifenschweiler, B. Sc. David Saleh, B. Sc. Yvonne Heneka.

Dr. rer. nat. Anke Neumann and Dr. rer. nat. Jens Rudat for their supportive attitude, comments during presentations, scientific input and ideas making lots of experiments more sophisticated.

Project co-workers Prof. Dr.-Ing. Matthias Franzreb and Dr.-Ing. Christian Morhardt for highly productive work on the project and scientific input beside of it.

The German Federal Ministry of Education and Research and Sanofi-Aventis Deutschland GmbH for funding the research project.

Markus Andre for his unlimited support, patience and love accompanying me throughout my work.

My parents Lübba Stolarow and Gennadij Stolarow за любовь, заботу и поддержку во всех моих начинаниях.

Preamble

Parts of this thesis are based on research and review articles, which have been previously peer-reviewed, published, submitted or under preparation for it. This dissertation includes results which have been generated between June 2012 and May 2015. Text parts taken from publications are indicated in full reference. Layout, citation style and reference formats have been adjusted to the layout in this thesis.

A detailed list of published works related to this thesis is included in the "Publications" section.

Chapter 1 gives an introduction on trypsin, its reaction mechanism and its applications. Furthermore, theoretical considerations of particle size and surface influencing the biocatalyst performance are depicted. Factors for estimation of biocatalyst performance are introduced. Examples from literature are given as a comparison of porous and non-porous biocatalyst carriers. This chapter contains parts of the publication:

Micromagnetic porous and non-porous biocatalyst carriers. (2014). Industrial Biocatalysis. P. Grunwald, Pan Stanford: pp. 521-552.

Chapter 2 includes an immobilization method development in organic medium for immobilization of trypsin on magnetic micro-particles. The chapter is based on the manuscript:

Immobilization of trypsin in organic and aqueous media for enzymatic peptide synthesis and hydrolysis reactions. (2015). BMC Biotechnology 15:77, pp. 1-8. Springer Open.

Chapter 3 contains considerations of particles surface and spacer size for modification of magnetic particles for further immobilization steps. The chapter is based on the submitted manuscript:

Effect of spacer modification on enzymatic synthetic and hydrolytic activities of immobilized trypsin. (2015). Article in press in Molecular Catalysis B: Enzymatic, Elsevier.

Chapter 4 presents a comparison of particle species of different size for application in a specifically developed peptide oligomerization reaction.

Publications related to this thesis

Original Research Paper and Book Chapters

Stolarow, J., Gerçe, B., Syldatk, C., Magario, I., Morhardt, C., Franzreb, M. and Hausmann, R. (2014)
Micromagnetic porous and non-porous biocatalyst carriers.
Industrial Biocatalysis. P. Grunwald, Pan Stanford: pp. 521-552.

Stolarow, J., Heinzelmann, M., Yeremchuk, W., Syldatk, C. and Hausmann, R. (2015)
Immobilization of trypsin in organic and aqueous media for enzymatic peptide synthesis and hydrolysis reactions.
BMC Biotechnology 15:77, Springer Open: pp. 1-8.

Andre, J., Saleh, D., Syldatk, C. and Hausmann, R. (2015) (in press)
Effect of spacer modification on enzymatic synthetic and hydrolytic activities of immobilized trypsin.
Molecular Catalysis B: Enzymatic, Elsevier.

Poster and Poster Presentations

Stolarow, J., Gerce, B., Syldatk, C. and Hausmann, R. (2012)
Enzymimmobilisierung an magnetischen Partikeln.
30. DECHEMA ProcessNet Jahrestagung 10.09.-13.09.2012, Karlsruhe, Germany.

Stolarow, J., Syldatk, C. and Hausmann, R. (2014)
Immobilization of proteases on micro-magnetic particles for the application in peptide synthesis.
3rd DECHEMA Summer School "Biotransformations", 24.08-27.08.2014, Bad Herrenalb, Germany.

Stolarow, J., Gerce, B., Syldatk, C. and Hausmann, R. (2014)
Immobilisierung von Proteasen an mikromagnetischen Partikeln zum Einsatz in Peptidsynthesereaktionen.
31. DECHEMA ProcessNet Jahrestagung, 30.09. - 02.10.2014, Aachen, Germany.

Abstract

Using enzymes as catalysts for chemical reactions is a suitable alternative to chemical catalysts for given stereo-, regio- and group-selectivity and mild reaction conditions. Enzymes are applied for production of a broad range of products from commodities, fine chemicals to specialities such as pharmaceutical precursors. However, the production of industrial enzymes and their purification may still comprise a large part of the fine chemicals and pharmaceuticals process budget. In order to make enzymatic conversions more competitive for industrial bulk and specialty chemicals production the biocatalysts must be stable and reusable over several conversion cycles. This may be achieved by immobilization of enzymes onto solid carriers or cross-linking enzymes into larger aggregates. Through these methods the separation of the biocatalyst is simplified and the catalyst may be recycled. Nevertheless, the fixation of the biocatalyst transfers the reaction from a homogenous into a heterogeneous environment, thereby changing the reaction characteristics. Moreover, the structure of large molecules such as proteins adjusts depending on the micro-environment it is placed and immobilization conditions. An altering effect on the enzymes' specificity and activity in a certain reaction sort may be induced. These aspects of immobilization must be taken into account when developing an immobilization method.

Trypsin is a proteolytic enzyme that is applied in hydrolysis and synthesis reactions of large and small peptides. The catalyzed reactions involve peptide bond formation/hydrolysis following basic amino acids such as arginine and lysine. It is usually produced heterologously followed by chromatographic purification steps. However, working with a proteolytic enzyme may lead to biocatalyst instability due to self-digestion, denaturation or subsequent by-product formation in the conversion process. For its applications and its shortcomings trypsin represents an interesting model enzyme for immobilization studies. Its fixation onto solid carriers may prevent self-digestion and improve reusability.

Among other carriers for immobilization, magnetic micro- and nano-scale particles feature a non-porous surface leading to better substrate-to-catalyst-diffusion, small particle size compared to many porous carriers giving a high specific surface area and superparamagnetic behavior improving the

separability from the bulk medium. These particles were chosen as main model carrier for investigations of trypsin immobilization.

In the present work the immobilization process of trypsin onto magnetic particles was investigated. The investigation aimed to obtain knowledge about the specificity and activity behavior upon trypsin immobilization. Given a standard immobilization method with low activity yield alternative immobilization methods were developed and tested in peptide synthesis and well established hydrolysis reactions and compared to the standard method. In particular, investigations on immobilization medium, particle surface characteristics and particles size were accomplished in this work. The influence of these major factors on enzymatic activity, reaction type specificity, reusability and by-product formation in di- and oligopeptide synthesis and hydrolysis reactions were evaluated.

Chapter 1 gives an introduction on trypsin, its reaction mechanism and its applications. Furthermore, theoretical considerations of particle size and surface influencing the biocatalyst performance are depicted. Factors for estimation of biocatalyst performance are introduced. Examples from literature are given as a comparison of porous and non-porous biocatalyst carriers.

In Chapter 2 “Evaluation of immobilization method” a dipeptide synthesis reaction catalyzed by trypsin as a model reaction for specificity and activity evaluation was developed. Within this reaction and a well known hydrolysis reaction the immobilization methods were compared. A newly developed immobilization method, by which trypsin is immobilized in micro-aqueous organic medium onto magnetic particles was compared to a standard method within buffer solution. For development of the new immobilization method a solvent screening and influence factor optimization were accomplished. Important influence factors such as water content in organic medium, particle activation and enzyme coupling time, indirect pH of trypsin lyophilizate, protein loading and substrate imprinting were identified. In synthesis reaction an up to sixfold improvement of specific enzyme activity compared to the standard method was achieved. However, in hydrolysis reaction both methods showed similar specific activities. From these results an important finding of this chapter was different specificity of trypsin in hydrolysis and synthesis depending on immobilization method. Furthermore, trypsin immobilized by the

organic method showed a good reusability with activity loss of 10% over ten sequential synthesis and hydrolysis cycles.

Chapter 3 “Evaluation of chemical nature of carrier and binding” is focused on investigations of spacer introduction on particle surface for subsequent trypsin binding and chemical binding nature of trypsin onto particles. Non-covalent affinity binding of trypsin yielded higher specific trypsin activity compared to covalent binding nature. Spacers of different length and functional groups were tested for particle and trypsin modification. Spacer length had a significant improving effect on specific synthesis activity of immobilized trypsin. However, a combination of immobilization in organic medium as developed in Chapter 2 with spacer binding did not achieve a significant total improving effect neither in synthesis nor in hydrolysis reaction types. The reusability of spacer bound trypsin reached over 70% in synthesis and over 50% in hydrolysis reactions.

Chapter 4 “Influence of particle size” aimed to compare different particle sizes for trypsin immobilization. A special focus was set upon product and by-product formation rates in a model reaction with peptide hydrolysis and synthesis steps as part-reactions. For this aim a peptide oligomerization reaction using Bz-Arg-OEt as acyl donor and Arg/Lys-OEt as nucleophiles was developed. One amino acid serves as monomer, which may be coupled by trypsin to oligomers with different polymerization degrees. By hydrolysis different oligomers steps may be further converted to more stable end products. A general reaction scheme was confirmed by product identification and monitoring the reaction kinetics with free enzyme. Arg-OEt appeared as a better nucleophile for synthesis product formation by trypsin than Lys-OEt. Trypsin immobilized on carriers with different particle size was used in oligomerization reaction. The used particles showed significant differences in hydrolysis and synthesis product reaction rates and maximum yields. The reaction rate of hydrolysis product formation was strongly decreasing with increasing particles size, while synthesis rates were affected when particle size exceeded 100 nm. For maximum hydrolysis product yield free trypsin and highly porous and large particles were shown to be most suitable, while non-porous middle-sized magnetic particles achieved highest maximum synthesis product yield. These results were the most interesting in this chapter. By immobilization a new tool for adjusting reaction rates and yields in production processes was found.

Zusammenfassung

Enzyme als Biokatalysatoren stellen aufgrund hoher Stereo-, Regio- und Gruppenselektivitäten und milden Reaktionsbedingungen eine gute Alternative zu chemischen Katalysatoren dar. Mithilfe von Enzymen kann ein breiter Bereich an Produkten von Basischemikalien, Feinchemikalien bis hin zu Spezialprodukten wie Vorstufen von pharmazeutischen Wirkstoffen abgedeckt werden. Die häufig schlechte Wirtschaftlichkeit enzymkatalysierter Prozesse aufgrund der hohen Herstellungskosten des Enzyms sowie die benötigte hohe Enzym- und Produktreinheit limitieren den Einsatz von Biokatalysatoren bei industriellen Anwendungen. Um die Konkurrenzfähigkeit der Biokatalysatoren gegenüber rein chemischen Verfahren zu verbessern, ist es daher notwendig diese stabiler und wiederverwendbar zu machen. Dies kann durch die Immobilisierung von Enzymen auf festen Trägern oder Quervernetzung untereinander bewältigt werden. Nichtsdestotrotz bewirkt der Immobilisierungsprozess eine Übertragung der Reaktion von homogenen zu heterogenen Bedingungen und verändert damit die Reaktionscharakteristik. Weiterhin passt sich die Struktur eines Makromoleküls wie die eines Proteins den Bedingungen in seiner Mikroumgebung an, wodurch ebenfalls veränderte Spezifität und Aktivität des Enzyms induziert werden können. Diese Aspekte müssen bei der Entwicklung einer Immobilisierungsmethode beachtet werden.
Trypsin ist ein proteolytisches Enzym, das bei Hydrolyse- und Synthesereaktionen von großen und kleinen Peptiden Verwendung findet. Die katalysierten Reaktionen sind spezifisch für eine Synthese/Hydrolyse von Peptidbindungen nach einer basischen Aminosäure wie Lysin oder Arginin. Das Arbeiten mit einem proteolytischen Enzym kann jedoch zur Instabilität des Biokatalysators durch Selbstverdau, Denaturierung oder Nebenproduktbildung bei der enzymatischen Umsetzung des Substrates führen. Aufgrund der Anwendungen in unterschiedlichen Reaktionstypen und seiner Nachteile stellt Trypsin ein interessantes Modellenzym für die Entwicklung einer Immobilisierungsmethode dar. Die Fixierung von Trypsin an einen festen Träger kann den Selbstverdau verhindern und die Wiederverwendbarkeit verbessern.
Unter vielen Trägerarten zur Enzymimmobilisierung zeichnen sich magnetische Mikro- und Nanopartikel durch eine unporöse Oberfläche, die zu

einem guten Substrattransport zum Enzym führt, eine im Vergleich zu porösen Trägern kleine Partikelgröße, die eine große spezifische Oberfläche bewirkt und ein superparamagnetisches Verhalten, das zu einer besseren Abtrennbarkeit vom Reaktionsmedium führt, aus. Diese Partikel wurden vornehmlich als Modellträger zur Untersuchung der Immobilisierung von Trypsin ausgewählt.

Im Rahmen der vorliegenden Arbeit wurden Immobilisierungsprozesse für Trypsin auf magnetischen Mikropartikeln untersucht, um Erkenntnisse zur Spezifität und Aktivität des immobilisierten Trypsins zu erlangen. Dazu wurde eine Standardimmobilisierungsmethode, die eine niedrige Aktivität von Trypsin bewirkt, mit einer neuen Methode mit modifizierten Immobilisierungsparametern verglichen. Der Vergleich sollte anhand der Aktivitätsmessung in einer Peptidsynthese- sowie einer etablierten Hydrolysereaktion erfolgen. Im Speziellen wurden in dieser Arbeit der Einfluss des Immobilisierungsmediums, der Partikeloberfläche und der Partikelgröße untersucht. Der Einfluss dieser Hauptfaktoren auf die Enzymaktivität, Spezifität der enzymatischen Reaktion, Wiederverwendbarkeit und Nebenproduktbildung in Di- und Oligopeptidsynthese- und Hydrolysereaktionen wurden evaluiert.

Kapitel 1 gibt eine Einführung zu Trypsin, dem Reaktionsmechanismus und seinen Anwendungen. Desweiteren wurden theoretische Betrachtungen der Partikelgröße und Oberfläche dargestellt. Einfluss- und Zielfaktoren zur Abschätzung der Leistung des Biokatalysators wurden eingeführt. Literaturbeispiele zum Vergleich von porösen und nicht-porösen Trägern für Biokatalysatoren wurden herangeführt.

In Kapitel 2 “Evaluation of immobilization method” wurde eine Trypsin-katalysierte Dipeptidsynthesereaktion als Modellreaktion zur Bestimmung der Spezifität und Aktivität entwickelt. Bei dieser Reaktionsart und einer weiteren bekannten Hydrolysereaktion von Trypsin wurden Immobilisierungsmethoden gegenübergestellt. Eine in dieser Arbeit neu entwickelte Immobilisierungsmethode, in der Trypsin im organischen Medium an magnetische Partikel gekoppelt wird, wurde mit einer etablierten Standardimmobilisierungsmethode in Puffermedium verglichen. Zuerst wurde ein Medienscreening mit mehreren Lösungsmitteln durchgeführt und die Methode in Hinsicht auf mehrere Einflussfaktoren optimiert. Als Haupteinflussfaktoren für die Immobilisierung im organischen Medium

wurden der Wasseranteil im organischen Lösungsmittel, die Dauer der Partikelaktivierung und Trypsinkopplung, der indirekte pH des Trypsinlyophilisats, die Proteinbeladung und Substratzugabe bei Trypsinkopplung identifiziert. In der Synthesereaktion wurde eine bis zu sechsfache Steigerung der spezifischen Aktivität des Trypsins aus der organischen Immobilisierung gegenüber der wässrigen Methode erreicht. In der Hydrolysereaktion jedoch, wurden ähnliche spezifische Aktivitäten für beide Methoden gemessen. Damit wurde eine wichtige Erkenntnis über den Einfluss der Immobilisierungsmethode auf die Spezifität des immobilisierten Enzyms gewonnen. Desweiteren zeigte organisch immobilisiertes Trypsin eine gute Wiederverwendbarkeit mit einem Aktivitätsverlust von 10% nach zehn aufeinanderfolgenden Hydrolyse- und Synthesezyklen.

Kapitel 3 “Evaluation of chemical nature of carrier and binding” enthält Untersuchungen zur Oberflächenmodifizierung der Partikel mittels Spacern und der Bindungsart des Trypsins an die Oberfläche. Nicht-kovalente Affinitätsbindung des Trypsins ergab eine höhere spezifische Aktivität gegenüber kovalenter Bindungsart. Spacer mit unterschiedlicher Länge und funktionellen Gruppen wurden zur Partikel- und Trypsinmodifizierung getestet. Die Spacerlänge hatte einen signifikant steigernden Effekt auf die spezifische Syntheseaktivität des immobilisierten Trypsins. Eine Kombination aus organischer Immobilisierungsmethode nach Kapitel 2 und Spacermodifizierung erbrachte jedoch weder in der Hydrolyse- noch in der Synthesereaktion einen steigernden Gesamteffekt auf die spezifische Aktivität. Die Wiederverwendbarkeit des Spacer-gebundenen Trypsins lag bei über 70% in der Synthese und bei über 50% in der Hydrolysereaktion.

Kapitel 4 “Influence of particle size” beschäftigt sich mit dem Vergleich unterschiedlicher Partikelgrößen bei der Trypsinimmobilisierung. Ein besonderes Augenmerk galt den Produkt- und Nebenproduktbildungsraten in einer Modellreaktion mit Peptidsynthese- und Hydrolyseschritten als Teilreaktionen. Dazu wurde eine Peptidoligomerisierungsreaktion mittels Trypsin unter Verwendung von Bz-Arg-OEt als Acyldonor und Arg/Lys-OEt als Nucleophilen entwickelt. Aminosäuremonomere können durch Trypsin zu Oligomeren unterschiedlichen Polymerisierungsgrades gekoppelt. Durch Hydrolyse können die entstandenen Oligomere zu stabileren Endprodukten umgewandelt werden. Ein allgemeiner Reaktionsablauf konnte durch Produktidentifizierung und kinetische Produktmessungen mit freiem Trypsin

bestätigt werden. Arg-OEt stellte im Vergleich zu Lys-OEt das bessere Nucleophil für die Bildung des Syntheseproduktes dar. Trypsin wurde auf Partikeln unterschiedlicher Größen immobilisiert und in der Oligomerisierungsreaktion eingesetzt. Die verwendeten Partikel zeigten signifikante Unterschiede in den Hydrolyse- und Syntheseaktivitäten und Maximalausbeuten. Die Bildungsrate des Hydrolyseproduktes nahm stark mit steigender Partikelgröße herab, während die Syntheserate erst ab einer Partikelgröße von 100 nm abfiel. Zum Erreichen der maximalen Hydrolyseproduktausbeute erwiesen sich freies Trypsin und große, poröse Partikel als geeignet, während nicht-poröse mittelgroße Partikel die höchsten Syntheseproduktausbeuten erzielten. Anhand dieser Ergebnisse konnte die Immobilisierung als ein neues Werkzeug zur Kontrolle von Reaktionsraten und Ausbeuten identifiziert werden.

Table of contents

1. Introduction

This chapter contains a part based on the publication

MICRO-MAGNETIC POROUS AND NON-POROUS BIOCATALYST CARRIERS

Julia Stolarow[a], Berna Gerçe[a], Christoph Syldatk[a], Ivana Magario[b], Christian Morhardt[c], Matthias Franzreb[c], Rudolf Hausmann[d]

[a]Institute of Process Engineering in Life Science, Section II: Technical Biology, Karlsruhe Institute of Technology, Karlsruhe, Germany [b]Investigación y Desarrollo en Technología Quimica (IDTQ), Grupo Vinculado PLAPIQUI-CONICET Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba. Córdoba, Argentina [c]Institute of Functional Interfaces, Process Technology of Particle-based Interfaces, Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, Germany [d]Department of Bioprocess Engineering, Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

---

The final publication is available at http://www.crcpress.com/

Bibliographic details:

Published in *Industrial Biocatalysis*, December 11, 2014, pp. 521 -552
Pan Stanford Publishing Pte. Ltd.

DOI:10.1201/b17828-15
Print ISBN 9789814463881 – eBook 9789814463898

Authors' contributions to the publication:

Julia Stolarow carried out the major literature review work, carried out the case study modeling, and wrote the manuscript.

Berna Gerçe proof read the manuscript.

Christoph Syldatk supervised the work and provided administrative support.

Ivana Magario provided research on modeling and theoretical background, and proof read the manuscript.

Christian Morhardt designed the magnetic separator scheme and proof read the manuscript.

Matthias Franzreb proof read the manuscript and provided theoretical support.

Rudolf Hausmann contributed to the design of the study, discussion of published research data, and revised the manuscript.

1.1. Using enzymes as biocatalysts

Enzymes offer several advantages compared to inorganic catalysts. They operate at mild conditions, are mostly evolved to be highly selective and feature high turnover numbers (Chen *et al.* 2005; Macario and Giordano 2013). Furthermore, considering "green" production processes they are highly bio-degradable (Illanes 2008). The reaction types catalyzed by enzymes comprise a wide range. According to the type of reaction, enzymes are divided into six enzyme classes with respective enzyme commission numbers (EC) constituted by the International Union of Biochemistry and Molecular Biology: EC 1 Oxidoreductases, EC 2 Transferases, EC 3 Hydrolases, EC 4 Lyases, EC 5 Isomerases, EC 6 Ligases.

Industrial application of enzymes benefits from the advantages stated above. Most commonly enzymes are applied in the production of pharmaceuticals, food and feed, cosmetics, fabrics, household products and biofuels (Figure 1.1) (DiCosimo *et al.* 2013).

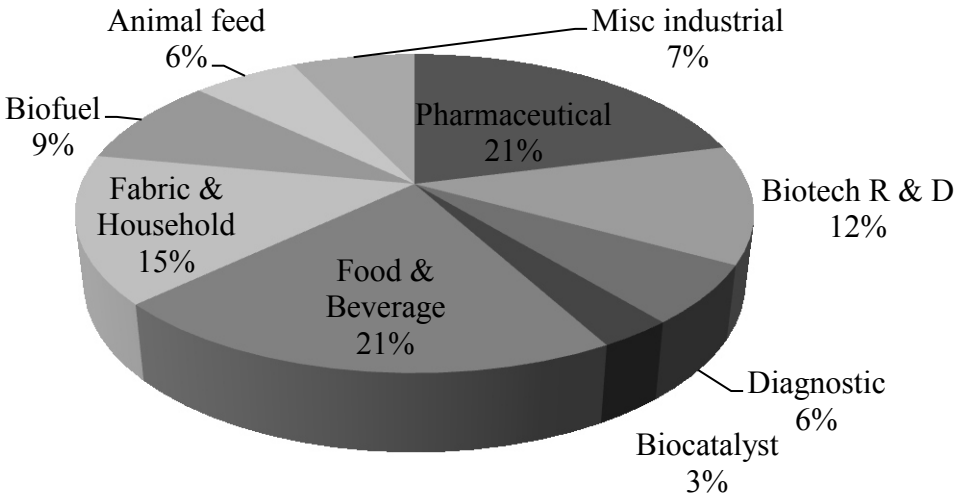


Figure 1.1. Industrial fields of enzyme application modified after DiCosimo (2013).

The distribution of enzyme classes in industrial applications is shown in Figure 1.2 (Liese *et al.* 2006). Hydrolases, which enable the transfer of groups to water, comprise the largest part of industrial enzymes. This fact is attributed to their group-, stereo- and regio-selectivity and the absence of a co-factor, which facilitate application on industrial scale. Hydrolases are sorted into 13 sub-classes depending on reaction bond-type they act on, e.g. ester, glucoside, peptide, ether or amide bonds. Despite the name, hydrolases are commonly used in synthesis reactions. These reactions are often executed at

non-physiological conditions, realized either under equilibrium or kinetic control.

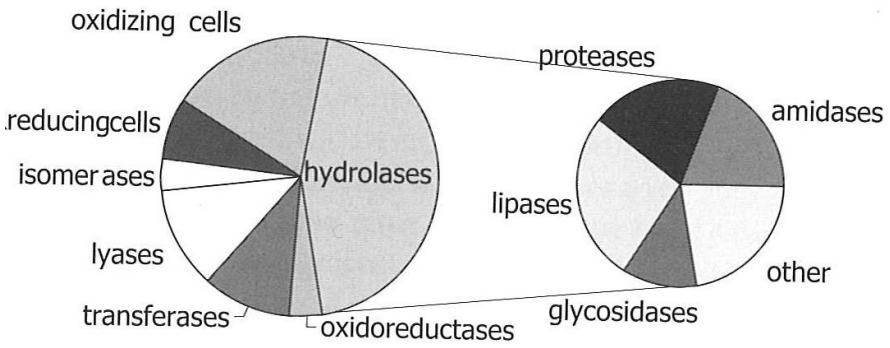


Figure 1.2. Enzyme classes used for industrial biotransformations extracted from Liese *et al.* (2006).

1.2. Proteases: mechanism and possible reactions

Peptidases or proteases (EC 3.4) are hydrolases that catalyze the formation and hydrolysis of amide links, for example between amino acids. They are further divided into endo-and exopeptidases depending on the respective cleavage site within or at the end of the peptide chain. Prominent representative of exoproteases is Carboxypeptidase B used for double cleavage of arginine from insulin B-chain resulting in human insulin (Liese *et al.* 2006). Endopeptidases feature several reaction mechanisms for cleavage or formation of peptide bonds and thus are further sorted into: serine, cysteine, metallo-, threonine, aspartate and unknown-mechanism-peptidases. Metallopeptidases contain a divalent metal ion coordinated in the protein backbone via ligands. Usually the metal ion is zinc, manganese or cobalt (Frey and Hegeman 2007). An industrially applied metalloprotease is thermolysin catalyzing the L-selective peptide bond formation between D,L-phenylalanine methyl ester and L-aspartic acid, which is further converted to the highly active sweetener aspartame. Threonine proteases contain a threonine residue within the active site. Aspartic proteases contain two conserved aspartates in their active site and are usually active at acidic pH. Pepsin is one representative of this group. Cystein proteases are dependent on a dyad of cysteine and histidine at active site. One example from this sub-group is papain which is broadly used as meat tenderizer (Rao *et al.* 1998).

Serine proteases are dependent on a triad of amino acids at active site consisting of serine 195, histidine 57 and aspartate 102. The hydroxyl side group of serine builds an H-bond towards imidazole ring of histidine which is

on the other side connected to aspartate through another H-bond (Figure 1.3). By this polarization the hydroxyl group of serine acts as a strong nucleophile and may perform an attack upon a carbonyl group of a target peptide bond (Figure 1.3). This tetrahedral intermediate state is stabilized by an oxyanion hole of the enzyme. In a subsequent step an acyl enzyme and an amino group of the target protein fragment stabilized by histidine through an H-bond occur. The amino group is released and the second part, the enzyme deacylation starts. Water replaces the amino component at histidine residue forming an H-bond. The OH-group of water performs a nucleophile attack upon the carbonyl group of the acyl-enzyme building again a tetrahedral product. The acyl-peptide fragment is released liberating the active site for a new reaction cycle. The major mechanism of proteases is complemented by the specificity for a certain cleaving position (Berg and Tymoczko 2012). The hydrophobic binding hole in S1 position is responsible for this specificity (Figure 1.4). Table 1.1 gives the preferential cleaving positions of several proteases (Rao *et al.* 1998). Prominent representatives of serine proteases are trypsin, chymotrypsin, subtilisin and elastase.

Table 1.1. Specificity of proteases for amino acids in P_n position (Rao *et al.* 1998).

Enzyme	Peptide bond cleaved[a]
Trypsin	-Lys (or Arg) ↓-----
Chymotrypsin, subtilisin	-Trp (or Tyr, Phe, Leu)↓------
Staphylococcus V8 protease	-Asp (or Glu)↓------
Papain	-Phe (or Val, Leu)-Xaa↓-----
Thermolysin	---↓- Leu (or Phe) ------
Pepsin	-Phe (or Tyr, Leu)↓-Trp (or Phe, Tyr)

[a] The arrow indicates the site of action of the protease. Xaa, any amino acid residue.

Trypsin belongs to the chymotrypsin-like protease family and is produced in the pancreas of many vertebrates. It is generated by proteolytic cleavage from a so called zymogen, an inactive precursor named trypsinogen. It fulfills digestive function in the duodenum and initiates the activation of several other hydrolases from zymogens such as chymotrypsin, elastase, carboxypeptidase and lipase (Berg and Tymoczko 2012). Trypsin is specific for peptide bonds following basic amino acids such as arginine and lysine at P1 position (Figure 1.4). Trypsin features an aspartate 189 in S1 position that is responsible for

this specificity (Kraut 1977). Porcine and bovine trypsin may be recombinantly produced in *Pichia pastoris*, *Aspergillus niger/A. oryzae* or *Escherichia coli* at industrial scale (Yee and Blanch 1993; Woldike and Kjeldsen 1999; Muller *et al.* 2007).

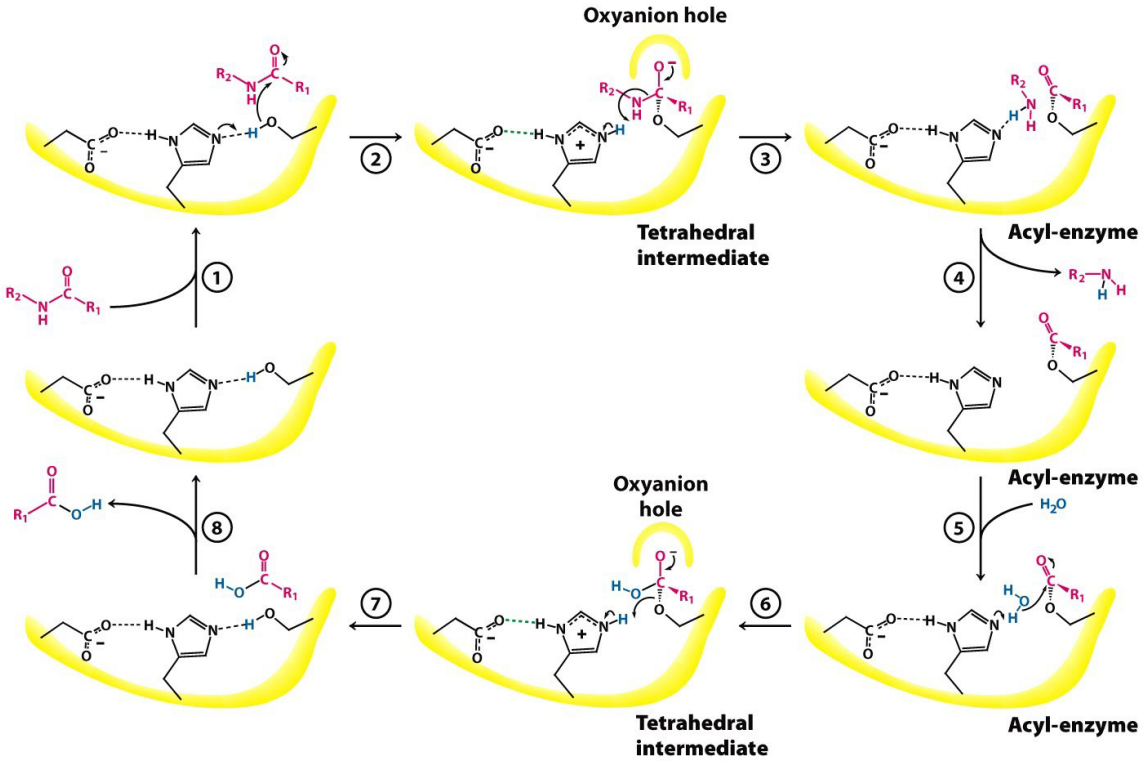


Figure 1.3. Mechanism of peptide hydrolysis by serine protease in an acid-base-catalyst principle. The reaction proceeds in eight steps: 1) substrate binding, 2) nucleophile attack of serine onto the carbonyl group of a peptide bond, 3) collapse of the tetrahedral intermediate, 4) release of the amino-component, 5) water addition, 6) nucleophile attack of water onto acyl-enzyme-intermediate, 7) collapse of the tetrahedral intermediate, 8) release of carbonic acid component extracted from (Berg and Tymoczko 2012).

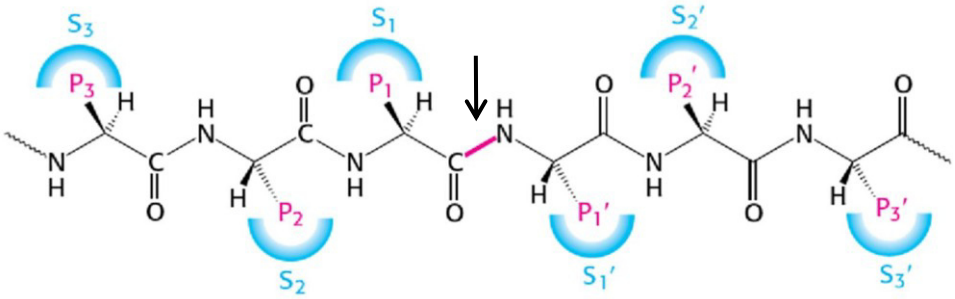


Figure 1.4. Protease specificity nomenclature in protease-substrate-interaction after Schechter and Berger (1967). The cleaved peptide bond is indicated by an arrow as reference point. The active site sub-sites of the protease are named S_n when they are situated on the left of the cleaved peptide bond and S_n' on the right hand of the peptide bond. The positions on the substrate peptide P are counted from the point of cleavage and thus have the same numbering as the enzyme subsites; extracted from Berg and Tymoczko (2012) (Schechter and Berger 1967; Berg and Tymoczko 2012).

It is industrially applied in insulin production from an insulin precursor where it cleaves a pre-chain and C-peptide and/or adds an amino acid-ester at the end of the insulin B-chain (Liese *et al.* 2006).

Furthermore, trypsin is applied in the production of polio and other vaccines. In the food industry trypsin may be used for digestion of allergenic proteins, flavoring of milk products, as baking enzyme or as meat tenderizer (Rao *et al.* 1998; Rawlings *et al.* 2007).

As mentioned above, the formation of an enzyme bond through a protease requires one of two possible strategies. Under equilibrium control the formation of amide bonds by proteases is implemented by water exclusion from the medium, high substrate concentrations or precipitation/immediate separation of the product.

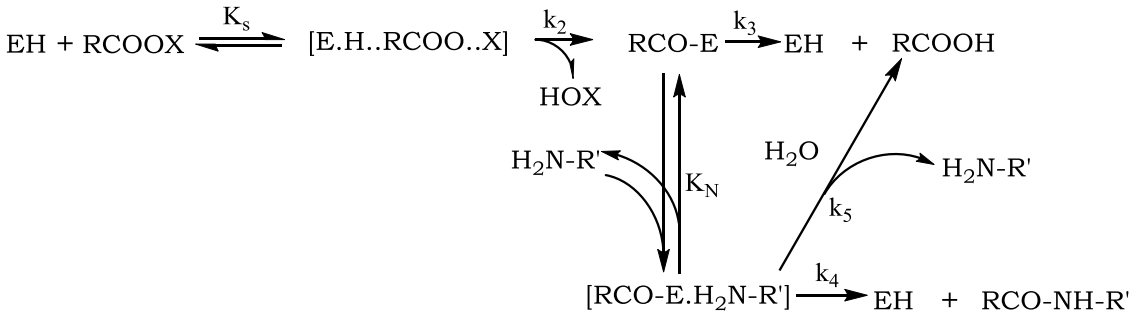


Figure 1.5. Serine- and cysteine-protease-catalyzed kinetically-controlled peptide synthesis. EH=protease, RCOOX- acyl donor (carboxyl component); H_2N-R'=amino component, X side chains of the ester leaving group extracted from (Schellenberger and Jakubke 1991).

Kinetically controlled peptide synthesis requires an activated carboxyl component (ester or amide). Under this condition the formation of an acyl-enzyme complex is fast, while the deacylation step with nucleophile (water or an amino-component) is the rate-limiting step. The kinetically-controlled approach requires serine or cysteine proteases, as only these may form an acyl-enzyme intermediate. The yield of the formed peptide is significantly influenced by the hydrolysis to aminolysis rate ratio (Figure 1.5) (Schellenberger and Jakubke 1991).

1.3. Aspects of industrial enzyme application

However, the industrial enzyme application necessitates high product yields, low by-product formation and low biocatalyst costs (Tufvesson *et al.* 2011). Although with the use of enzymes the selectivity for substrates/products is increased, there are several barriers to enzymatic application in protein synthesis. Hydrolysis of the acyl-enzyme complex may compete with desired

synthesis reaction or product cleavage may lower the product yield. The specificity of proteases for a certain cleavage position does not allow for a universal biocatalyst applicable on all sorts of substrates. Furthermore, many proteases feature cleaving sites within their own molecule chain leading to autoproteolysis (self cleavage) when the enzyme is solubilized in substrate solution. This may result in inefficient process conduction.

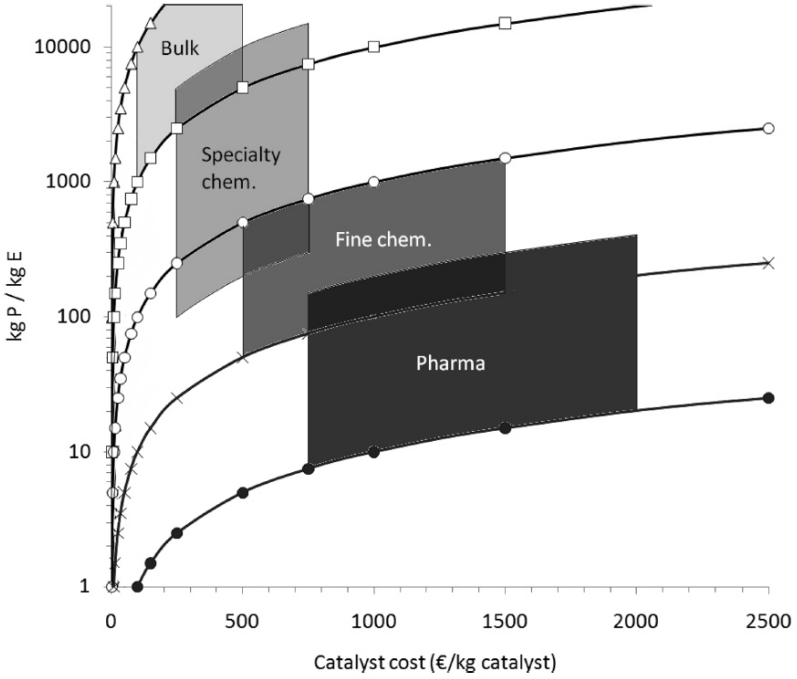


Figure 1.6. Biocatalyst cost effect on the requirements for biocatalyst productivity expressed as kg of product per kg of biocatalyst used for production of bulk, fuel, or speciality chemicals with immobilized enzymes. The allowable cost contribution for each sector was considered: △ 0.01 €/kg; □ 0.1 €/kg; ○ 1 €/kg; × 10 €/kg, ● 100 €/kg; extracted from Tufvesson *et al.* (2011).

Moreover, when the enzyme is located in the reaction medium, a separation or deactivation of the enzyme is required in order to control the process and obtain purified products. The required steps increase the costs of a process significantly. The allowable costs of an enzyme differ depending on the field of application. Figure 1.6 gives a correlation between biocatalysts costs and required productivity under consideration of allowable cost contribution of an enzyme (Tufvesson *et al.* 2011). However, one possibility of reducing the costs of a biocatalyst is the increase of batches, which may be conducted under catalyst reuse (Cao *et al.* 2003; Sheldon 2007). This process optimization may be achieved by immobilizing the biocatalyst onto a carrier. The immobilization techniques comprise special impact factors for optimization of biocatalytical production processes.

The next sub-chapter gives a comprehensive description of benefits and drawbacks of magnetic biocatalyst carriers.

1.4. Micro-magnetic porous and non-porous biocatalyst carriers

Immobilized biocatalysts offer several benefits in comparison to the application of soluble free enzymes or free cells. A biocatalyst bound to a solid surface allows for its reutilization and its use in continuously operated reactors such as columns or stirred-tank reactors is facilitated. Additionally, the immobilization of a biocatalyst also enables an easier separation from the media and downstream-processing. When using purified enzymes, the costs often constitute a significant part of the total production process. For this reason, recycling of the immobilized biocatalyst may lead to an overall cost benefit. Magnetic micro- and nano-carriers are known for many years in standard applications such as medicine, bioscience and bioseparation as well as purification processes. These sorts of solid particles are commonly used in such methods as immunodetection, genetic transformation, bioanalytics, magnetic resonance imaging and drug delivery. In contrast to these well known mostly biomedical applications using magnetic micro- and nano-supports in biocatalysts immobilization is a developing field which needs to be further explored. Featuring several interesting qualities the development of new applications gives promising solutions for downstream- and enzymatic processes. Magnetic micro- and nano-carrier systems offer a comparably large specific surface area due to their small size between 10 nm and 10 µm. The magnetic nature offers a simple solution to post-reaction separation processes. However, in contrast to relatively larger porous particles they mostly feature compact non-porous surfaces. This facilitates the transport kinetics in comparison to porous carriers. For the evaluation of this potential benefits several enzyme and reaction-specific aspects have to be considered. Activity yield, recyclability and stability are the three basic considerations concerning the utilization of immobilized biocatalysts. An important point affecting all these aspects is the choice of the carrier.

Carriers for enzyme immobilization are characterized by their physical, chemical, geometrical and mechanical properties. Relevant physical and geometrical properties of the solid are shape, average size, particle size distribution, specific area, compressibility, adsorption of media compounds, pore size and pore size distribution, abrasion in stirred tank or fluidized beds reactors and swelling properties. Chemical characterization of carriers

includes degree of hydrophobicity, chemical, microbial and thermal stability. Carriers should be provided with sufficient elasticity and pressure resistance (Buchholz *et al.* 2005). However, one of the most important attributes of a carrier is its size.

Immobilization techniques can be assorted in three main groups: adsorptive, covalent binding and entrapment. Adsorptive techniques include interactions between the enzyme and the surface of the solid such as metal chelate binding, ionic binding and adsorption by van der Waals forces, hydrogen bonding or dipole-dipole forces. Within covalent binding, the enzyme is attached to the surface of a particle, incorporated inside a support by covalent linkages or cross-linked with other enzyme units or other proteins. Finally enzymes may be mechanically entrapped within a gel, membrane or a micro-capsule (Tischer and Kasche 1999).

Important performance criteria for immobilized enzymes are the space-time yield at process conditions, the intrinsic enzymatic activity, the stationary and operational effectiveness factor (Kasche 1983). The overall reaction rate under immobilized catalyst conditions is influenced by the catalytic reaction rate on carrier surface and by the mass transfer of substrates to the biocatalyst. Among different carrier types ones with porous and non-porous surfaces can be distinguished. Porous carrier morphologies offer a large specific surface area for enzyme attachment. On the other hand, mass transfer effects may negatively influence the overall reaction rate.

Micro-magnetic particles have been used since the 1970s for enzyme immobilization (Robinson *et al.* 1973). Mostly used magnetic materials are magnetite Fe_3O_4, ferrite Fe_2O_3, maghemite γ-Fe_2O_3 as well as cobalt Co, nickel Ni and nickel oxide NiO. Small nano-particles (approx. < 10 nm) of these materials expose superparamagnetic qualities. Each nano-particle becomes a single magnetic domain and shows superparamagnetic behavior when the temperature is above the so-called blocking temperature. Such individual nano-particles show a fast response to applied magnetic fields with negligible remanence (residual magnetism) and coercivity (the field required to bring the magnetization to zero). The risk of forming particle agglomerates is relatively low at room temperature (Lu *et al.* 2007). Superparamagnetic magnetite nano-particles range from approx. 3 to 15 nm in diameter. Often these materials are coated with polyethyleneimine, polyacrolein, polyacrylamide, polyvinyl alcohol, polymethyl methacrylate, silan, chitosan or alginate. The metal or metal-oxide

compounds can be situated in the core of a particle surrounded by the polymer, on the outside of the particle or distributed within the polymer. The coating may increase the particle size up to several µm and rarely up to 100 µm and results in different support appearance (Figure 1.7).

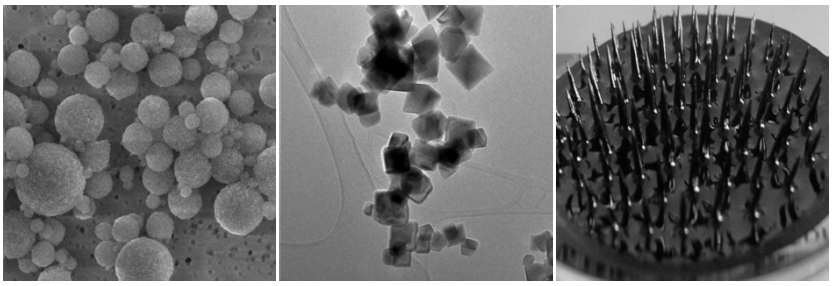


Figure 1.7. Examples for size-dependant appearance of different magnetic particles from left to right: polymer particles with trapped magnetite 1-10 µm, magnetic nano-composite 50-100 nm, 1-10 nm magnetite crystals suspended in a magnetic fluid.

The magnetically responsive nature allows for the selective manipulation and separation of biocatalyst coated particles in presence of other solution ingredients. Biocatalysts (e.g. enzymes or ribozymes) bound to micro-magnetic particles facilitate their use in industrial bioconversions processes. It becomes possible to magnetically separate the biocatalyst out of crude biological process liquors or defined reaction mixtures. By using magnetic biocatalyst carriers in this way, the several stages of sample treatment for biocatalyst separation (especially centrifugation, filtration and membrane separation) may be eliminated. In a further step a second sub-ordered or subsequent process can be initiated by recycling the biocatalyst bound particles (Figure 1.8).

Among magnetic enzyme carriers porous as well as non-porous surfaces are used. Generally, non-porous supports in the µm scale offer several potential advantages apart from mass transfer. They should be less susceptible to fouling which results in restriction of catalytic activity. The removal of fouling materials deposited within the pores of a conventional porous support is very difficult compared to those on the external surface. For their size and their solid core, non-porous supports should be more resistant to vigorous mixing (Halling and Dunnill 1980).

These qualities make non-porous micro magnetic particles interesting for applications in pharmaceutical production, synthesis of bulk or fine chemicals as well as degradation of toxic environmental compounds or recycling of valuable resources.

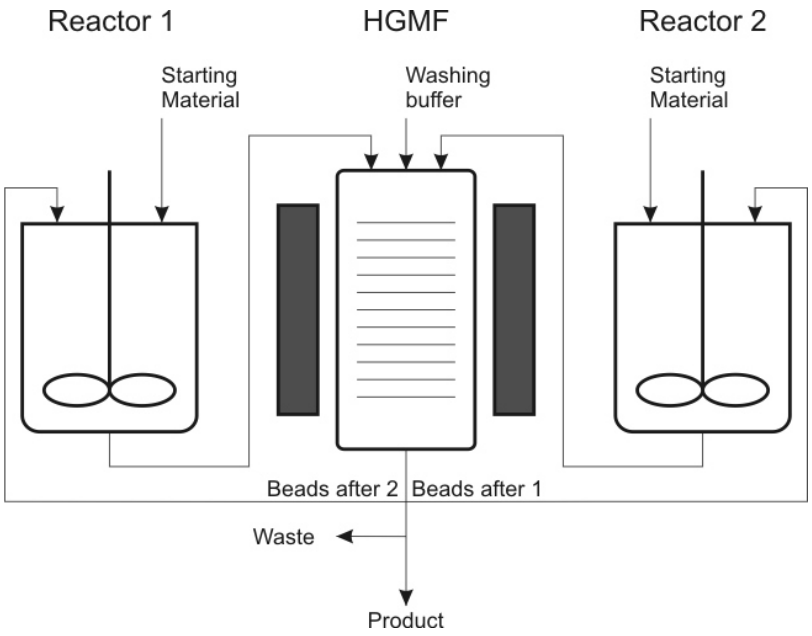


Figure 1.8. Process scheme of a magnetic particles batch and particle separation by high gradient magnetic separation (HGMF). The process starts in Reactor 1 by mixing the magnetic beads with the relevant starting material. After the binding of target biocatalyst the process slurry is pumped into the HGMF where an external magnetic field is applied. The beads are separated on stainless steel perforated discs and the supernatant is removed (Waste). The beads are washed with washing buffer. Afterwards the external magnetic field is switched off and the beads are pumped into Reactor 2 where the actual bioconversion process is taking place. Again a separation of magnetic particles by HGMF permits product separation from the biocatalyst. By this design a semi-continuous process is possible.

One of the first reviews on the topic of magnetic supports for biological compounds was compiled by Halling and Dunhill (1980), where coupling methods, surface properties and particle size is discussed in respect to their advantages and disadvantages in enzyme immobilization and bioaffinity adsorption. In recent past a number of reviews on the same topic were published referring to different aspects of this topic. Koneracka *et al.* (2006) focus on detailed immobilization procedures for proteins and enzymes onto various magnetic carriers. Several methods are given to verify the outcome of immobilization procedures and the properties of freshly prepared supports. In 2006 Kim *et al.* illustrate the stabilization mechanisms behind diverse enzyme immobilization approaches such as encapsulation, adsorption, covalent attachment and the combination of these on nanostructures. In 2012 Netto *et al.* describe a multitude of enzymes which have been immobilized onto superparamagnetic nanoparticles (Halling and Dunnill 1980; Kim *et al.* 2006; Koneracka *et al.* 2006; Netto *et al.* 2012).

So far the methods involving utilization of micro- and nano-magnetic particles are carried out on laboratory scale mostly in biomedical applications. For evaluation of processes for potential use on industrial scale in biocatalysis

further factors such as mass transport limitations must be considered. This chapter focuses on overall reaction rate properties of micro-magnetic non-porous carriers in comparison to porous supports as enzyme carriers intending to mark out their respective properties. Also carrier comparison examples from literature based on the influence of substrate transfer to and within the carrier surface in the heterogeneous reaction are discussed.

1.4.1. Theoretical considerations of mass transfer phenomena

The overall reaction kinetics for all heterogeneous catalyzed chemical reactions is determined by a combination of the intrinsic reaction kinetic and mass transfer phenomena. These mass transfer phenomena always comprise external mass transfer and additionally intern mass transfer when using porous catalysts. The mass transfer rate depends on the substrate concentration and the diffusion coefficient. The substrate concentration is relevant as it determines the maximum concentration gradient that drives the diffusion. Low molecular weight substrates generally combine high solubilities with high diffusion coefficients resulting in an advantageous situation. Specifically, for biological systems, however, often high molecular weight substrates are applied that display low solubility and low diffusion coefficients. Thus, in such systems the use of immobilized biocatalysts may lead to significant mass transfer limitations. As a result a reduced intrinsic activity of immobilized enzymes is apparent, the effect being more pronounced when using larger particles.

A relevant parameter for describing whether reaction kinetics is affected by mass transfer limitations is the effectiveness factor η of immobilized biocatalysts. This factor is defined as the relation of the specific enzyme activity ν immobilized on a carrier to the specific activity of the respective free enzyme. In order to estimate expected maximum effectiveness of an immobilized biocatalyst a mathematical modeling may be a time saving orientation tool. Theoretical carrier activities in dependency of protein loading may be estimated considering substrate transport effects on the overall reaction rate. The diffusion-reaction model for immobilized biocatalysts on porous and non-porous particles implies four general assumptions (Engasser and Horvath 1973; Chaplin and Bucke 1990):

1) Dynamic equilibrium (steady-state condition) for enzymatic reaction

and substrate transport between particle phase and fluid solution

2) the system is considered isothermal
3) one-dimensional substrate diffusion in particle film thickness described by the Fick's law
4) absence of any substrate partitioning or electrostatic effects at the particle surface

The effectiveness factor η of immobilized biocatalysts is calculated according to its definition as the ratio of the specific enzyme activity v immobilized on a carrier to the specific activity of the respective free enzyme and may be experimentally determined.

$$\eta = \frac{v_{immob}}{v_{free}} \tag{1-1}$$

This factor may also be interpreted as the ratio of the actual reduced reaction rate to the theoretical rate of reaction without mass transfer limitations.

For the calculation of the effectiveness factor the so called Thiele modulus has to be determined. The Thiele modulus Φ (equation 1-2) is a dimensionless number representing the relation between reaction rate and substrate diffusion rate to the catalyst within a porous support (Thiele 1939). This equation is generally formed as following:

$$\Phi = L \cdot \sqrt{\frac{A_s}{D}} \sim \frac{reaction\ rate}{diffusion\ rate} \tag{1-2}$$

Here the Thiele modulus is obtained from the combination of the carrier characteristic length L (ratio of carrier volume and carrier surface area), surface activity of the carrier (A_S) and the molecular diffusion coefficient D of the substrate in regard to pore geometry.
For enzymatic reactions assuming Michaelis-Menten kinetics and spherical porous carriers the Thiele modulus can be expressed as (Engasser and Horvath 1973; Atkinson and Davies 1974; Clark and Bailey 2002):

$$\Phi = \frac{R}{3} \cdot \sqrt{\frac{V_{max}}{D_{eff} \cdot K_m}} \tag{1-3}$$

In this equation R is the particle radius, V_{max} is the intrinsic reaction rate per unit reaction volume which is set in relation to diffusion of the substrate to the particle and K_m is the Michaelis-Menten constant. D_{eff} is the effective diffusion coefficient in porous structures defined as (Walas 1997):

$$D_{eff} = D \cdot \frac{\varepsilon}{\tau} \tag{1-4}$$

D is the diffusion coefficient of the substrate in the bulk fluid, ε is the porosity of the support and τ is the tortuosity of the pores. The tortuosity accounts for the curvature of a pore resulting in a longer distance traversed in the pores. According to the Stokes-Einstein equation the specific size of a substrate molecule destines its mobility towards the biocatalyst. For high molecular weight substrates D_{eff} becomes a crucial factor in the overall reaction rate.

In order to consider the nonlinear kinetics the ratio of the reaction rate and the Michaelis-Menten constant (V_{max}/K_m) is expressed in dependency of the substrate concentration (Kasche 1983):

$$\frac{V_{max}}{K_m} = v_P(s_0) \cdot q_P \cdot \rho \cdot \frac{(1+\beta_0)}{s_0} \tag{1-5}$$

In the above equation (1-5) ν is the protein specific activity at a given substrate concentration s_0 at particle surface. The protein loading of the carrier is given as q_p [mg/g] and β_0 is the dimensionless substrate concentration at the particle surface defined as s_0 / K_m (Kasche 1983).

The theoretical effectiveness factor is derived by the mathematical calculation of the reaction kinetics in all local positions within the pores of the particle and successive determination of the average value. This average value of the reaction kinetics is then divided by the maximum obtainable value without mass transfer limitation. An analytical solution for Michaelis-Menten kinetics is not possible, however, an approximation of effectiveness is given by following equations (Atkinson and Davies 1974):

$$\eta^{in}(\Phi, \beta_0) = \begin{cases} 1 - \dfrac{tanh\,\Phi}{\Phi} \cdot \left[\dfrac{1/\eta_D}{tanh\,(1/\eta_D)} - 1\right] : \dfrac{1}{\eta_D} \leq 1 \\ \eta_D - \dfrac{tanh\,\Phi}{\Phi} \cdot \left[\dfrac{1}{tanh\,(1/\eta_D)} - 1\right] : \dfrac{1}{\eta_D} > 1 \end{cases} \tag{1-6}$$

$$\eta_D = \sqrt{2} \cdot \left(\frac{1+\beta_0}{\Phi \cdot \beta_0}\right) \cdot [\beta_0 - \ln(1+\beta_0)]^{1/2} \tag{1-7}$$

These equations can be applied to the standard case in industrial biocatalysis where an excess of substrate ($s_0 >> K_m$) in bulk fluid is desirable and thus $\beta_0 >> 1$ (Figure 1.9). A negligible effect of diffusion resistance is maintained up to $\Phi \approx 3$, while a rapid decrease in effectiveness factor is obtained when $\Phi > 3$.

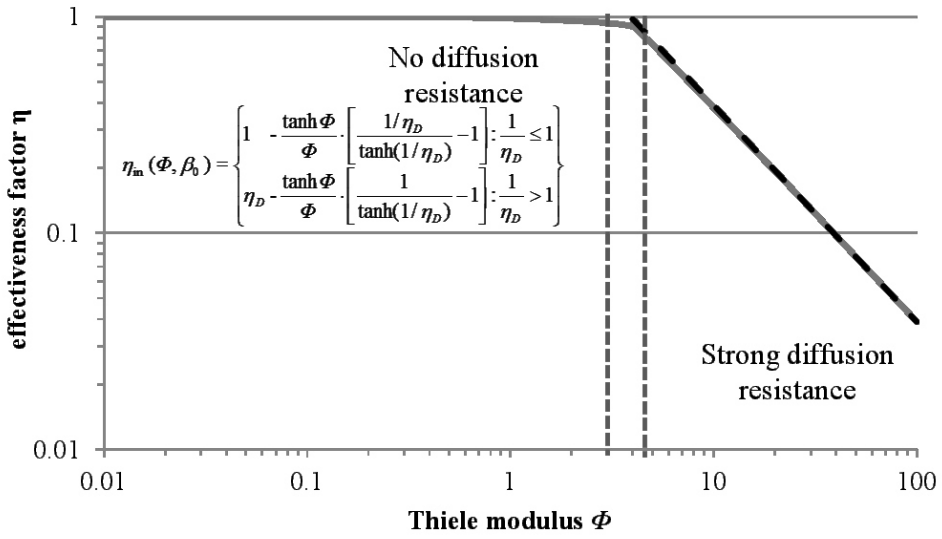


Figure 1.9. Theoretical effectiveness factor for porous carriers versus Thiele modulus at $s_0 > K_m$ resulting in dimensionless substrate concentration $\beta_0 = 10$.

This approximation of effectiveness (equations 1-6 and 1-7) is valid for spherical particles although only at relative small Thiele modulus values (< 1) (Yamane 1981). In some biocatalyzed processes the substrate is poorly soluble or inactivates the biocatalyst at high concentrations. Also if the particle radius is proportionally large, the substrate concentration in direct adjacency of the biocatalyst is much lower than in the bulk fluid. This leads to apparent substrate concentration lower K_m, $s_0 \leq K_m$. For such conversions, where β_0 approaches zero, η converges to the effectiveness factor of the corresponding first-order reaction. In this case the expression can be derived analytically as follows (Thiele 1939):

$$\lim_{\beta \to 0} \eta^{in} = \frac{3}{\Phi} \cdot \left(\frac{1}{tanh\,\Phi} - \frac{1}{\Phi} \right) \tag{1-8}$$

For $\beta_0 \rightarrow 0$ the transitional range for effectiveness factor with increasing Thiele modulus Φ is larger than above. The decrease occurs rapidly for Thiele modulus numbers higher $\Phi \approx 2$ (Figure 1.10).

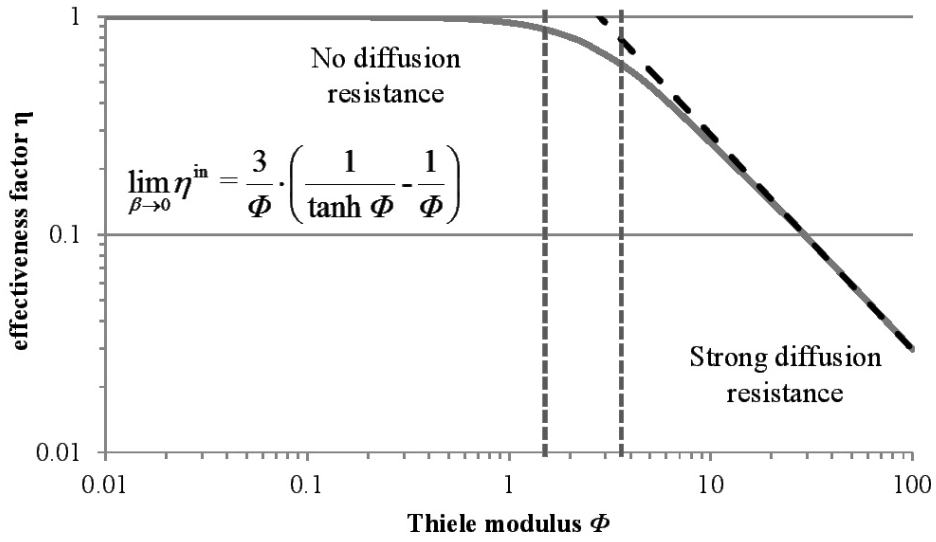


Figure 1.10. Theoretical effectiveness factor for porous carriers versus Thiele modulus for first order reactions in which substrate concentration s_0 is lower K_m, $\beta_0 \rightarrow 0$.

For applying the above equations (1-6, 1-7 and 1-8) it has to be further assumed that:

1) the protein is uniformly distributed within the porous particle,
2) there is no external diffusion limitation the substrate concentration at particle surface (β) being equal to the substrate concentration at the bulk (= β_b) and
3) the effective diffusion coefficient D_{eff} is constant within the particle.

Thus, provided that the intrinsic enzyme activity (v_P) and the effective diffusion coefficient (D_{eff}) are known, the reaction effectiveness factor can be estimated for every porous support in dependency of the protein loading.
In the above equations the assumption of no external diffusion limitations was made. In general the overall reaction rate of porous biocatalysts is affected by both external and internal mass transfer limitations. This overall reaction rate can be assessed by the Biot number *Bi* which represents the ratio of external mass transport to internal diffusion (Truskey *et al.* 2004):

$$Bi = \frac{k \cdot L}{D_{eff}} \tag{1-9}$$

In equation (1-9) k represents the mass transfer coefficient [units length/units time] while L is the characteristic length. The effectiveness factor considering external and internal mass transfer in first-order reactions is expressed as:

$$\eta = \frac{tanh\,\Phi}{\Phi\left[\frac{\Phi}{Bi}tanh\,\Phi + 1\right]} \tag{1-10}$$

For $Bi \rightarrow \infty$ the external mass transfer limitation becomes negligible when substrate concentration in bulk solution equals substrate concentration on the biocatalysts surface $s_0 = s_b$. In other case, when the external mass transport is highly significant for $Bi << 1$ the equation reduces to $\eta \approx Bi/\Phi^2$.

As the Thiele modulus is difficult to determine from experiments, a characteristic number was defined by Wagner, Weisz and Wheeler which includes only observable and measurable quantities (Wheeler 1951; Weisz and Hicks 1962; Weisz 1973):

$$W = \Phi^2 \cdot \eta = R^2 \frac{(-r/s_0)_{obs}}{D_{eff}} \tag{1-11}$$

In equation (1-11) r is the observed reaction rate, while s_0 represents the concentration of the substrate in bulk solution. With this modulus finding pore resistance effects from experiment is simpler.

When reactant fully penetrates the particle and satiates all its surfaces, the particle is in the diffusion free regime. This occurs when $\Phi < 0.4$ or $W < 0.15$. At the other extreme when substrate concentration is too low for the substrate to penetrate the center of the particle and the catalyst becomes unused then the particle is in strong pore resistance regime. This occurs when $\Phi > 4$ or $W > 4$.

For micro-magnetic non-porous particles the enzymatic conversion occurs due to the smooth surface only on the outer of the carrier. The Damköhler number second order gives this relation for extrinsic mass transfer (Damköhler 1937).

$$Da_{II} = R^2 \cdot \frac{V_{max}}{D \cdot K_m} \tag{1-12}$$

The particle characteristics that should be accounted for in these phenomena are the particle radius R, the substrate diffusion constant D and the enzymatic reaction model according to Michaelis and Menten with characteristic values of V_{max} and K_m.

For Michaelis-Menten reaction kinetics the external effectiveness factor can be derived from the steady state mass balance equations. Just as for porous carriers a favourable excess of substrate is assumed $s_0 > K_m$ (Chaplin and Bucke 1990; Kheirolomoom *et al.* 2002):

$$\eta^{ex} = \frac{(1+\beta_b)\cdot\left(\varphi - \sqrt{\zeta}\right)}{2\cdot\beta_b\cdot Da_{II}} \tag{1-13}$$

$$\varphi = 1 + \beta_b + Da_{II} \tag{1-14}$$

$$\zeta = \varphi^2 - 4\cdot\beta_b\cdot Da_{II} \tag{1-15}$$

In the above model the working range with no diffusion resistance lies at $Da_{II} \approx 6$ and the regime of strong diffusion resistance starts approximately at $Da_{II} \approx 20$ (Figure 1.11).

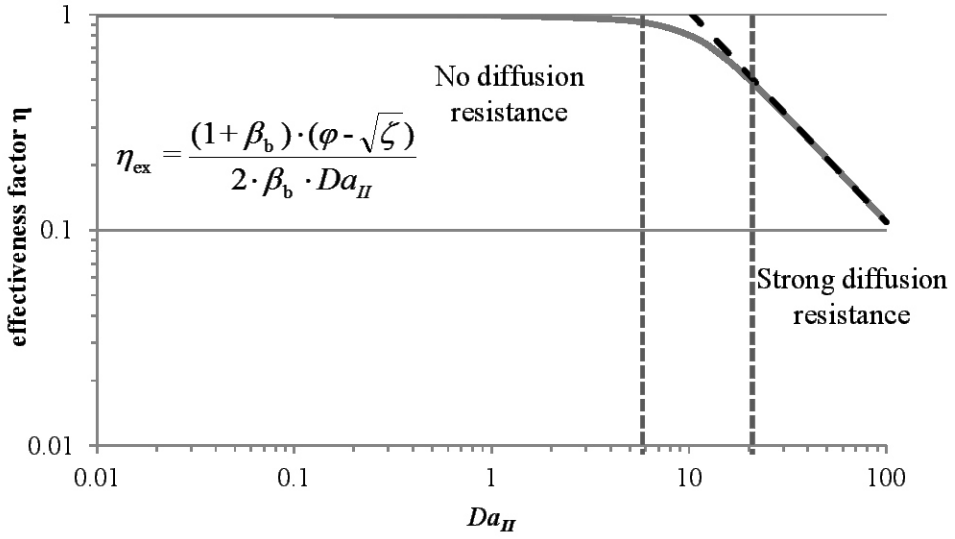


Figure 1.11. Theoretical effectiveness factor for non-porous carriers versus Damköhler number at substrate excess $\beta_0 = 10$.

In case of $s_0 \leq K_m$, where β_0 approaches zero, the Damköhler number can be simplified to a first order kinetic model, where K_m is not accounted. From this the effectiveness factor η will be described for first order extrinsic mass transfer kinetics as follows (Chaplin and Bucke 1990):

$$\eta = \frac{1}{1 + Da_{II}} \tag{1-16}$$

For the above case a rapid decrease in effectiveness factor with increasing Da_{II} takes place (Figure 1.12):

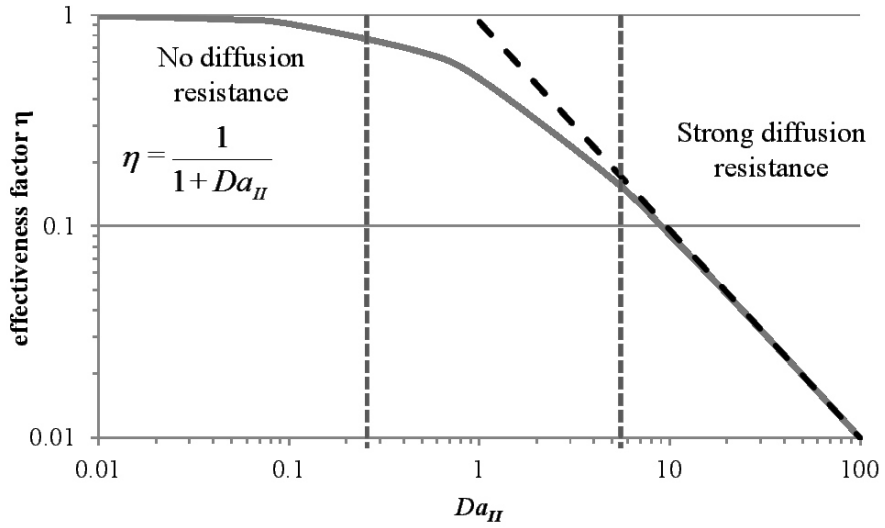


Figure 1.12. Theoretical effectiveness factor for non-porous carriers versus Damköhler number for reactions in which substrate concentration s_0 is lower K_m, $\beta_0 \rightarrow 0$.

In these particular equations the assumption was made, that the mass transfer of the substrate to the particle is driven only by diffusion. The thickness δ of the unstirred diffusion layer characterizes the concentration gradient that drives the diffusion velocity. The thickness of this layer can be reduced by stirring, which increases the velocity of the particles relative to the solvent. On the other hand the stirring causes shear forces that may cause abrasion of the particles, and thus the stirring speed has an upper limit in order to avoid this abrasion (Buchholz *et al.* 2005). For a stirred carrier suspension with Reynolds numbers $Re_p > 1$ the following correlation can be applied (Herndl and Mersmann 1981; Hoffmann 2003)

$$Sh = 2 + 0.991 \cdot Re_p^{1/3} \cdot Sc^{1/3} \tag{1-17}$$

In this equation *Sc* stands for Schmidt number and describes the ratio of viscosity to mass diffusivity. For very small non-porous magnetic carriers the value of Reynolds number Re_p tends to zero and the Sherwood number *Sh* approaches its minimal value of 2.

In order to evaluate the performance of a chosen support within a certain reaction type in terms of diffusion limitations, a modeling of the effectiveness factor in correlation to enzyme loading can be implemented. This modeling gives a prediction of the immobilizate's behavior. The evaluation of the real facts is confirmed or disproven by experimental data. A prediction of effectiveness factor under given working conditions for porous and non-porous spherical particles is given in Figure 1.13 and Figure 1.14.

For reaction rates abundantly higher than mass transport rate to the biocatalyst the non-porous carrier bound biocatalyst is clearly at advantage over pore-bound biocatalyst. The overall reaction kinetics is mainly controlled by mass transport limitations into the pore as the reaction is faster than the diffusion. This phenomenon is depicted in Figure 1.13. A simulation for given reaction and carrier characteristics such as particle radius R, molecular diffusion constant D and D_{eff} and specific enzyme activity v_{free} by assuming substrate concentration s_0 lower K_m and therefore $\beta_b \rightarrow 0$ was made. The effectiveness factor for porous supports with reaction kinetics faster than mass transport kinetics a rapid decrease with increasing protein loadings takes place, while for non-porous supports the effectiveness factor remains at the highest throughout progressing protein loading.

For processes where the reaction rate does not exceed the mass transport rate the difference between porous and non-porous carriers becomes smaller. As shown in Figure 1.14 the reaction is the overall reaction rate-limiting factor and the porous carrier's utilization becomes less disadvantageous.

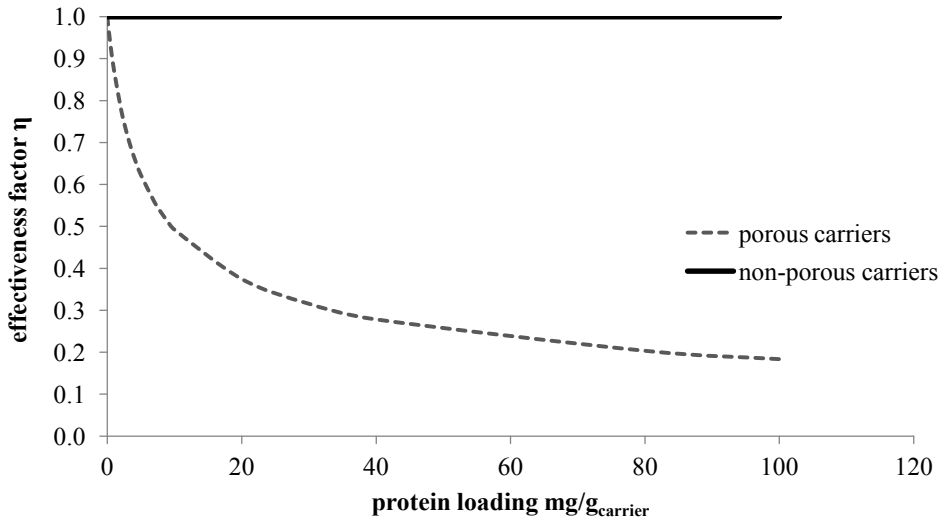


Figure 1.13. Effectiveness factor η versus protein loading displaying the influence of internal and external mass transfer effects within reaction rate >> mass transport rate enzymatic reactions with exemplary model parameters $\beta_0 \rightarrow 0$: porous support: R = 100 µm, D_{eff} = $6 \cdot 10^{-11}$ m²/s and v_{free} = 20 U/mg$_{Protein}$; non-porous support: R = 1 µm, D = $3{,}7 \cdot 10^{-10}$ m²/s and v_{free} = 20 U/mg$_{Protein}$.

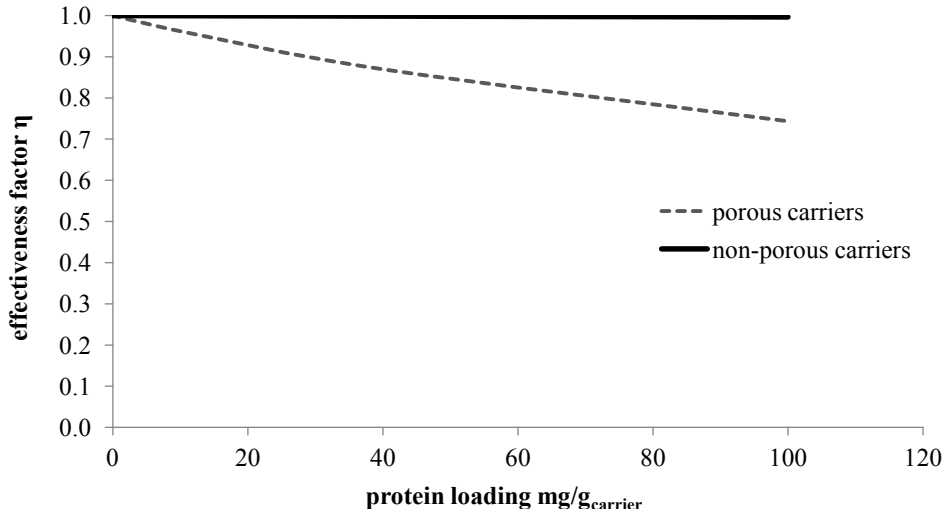


Figure 1.14. Effectiveness factor η versus protein loading displaying the influence of internal and external mass transfer effects within reaction rate ≤ mass transport rate enzymatic reactions with exemplary model parameters $\beta_0 \rightarrow 0$: porous support: R = 100 µm, D_{eff} = 3·10-11 m²/s and ν_{free} = 2 U/mg$_{Protein}$; non-porous support: R = 1 µm, D = 2·10-10 m²/s and ν_{free} = 2 U/mg$_{Protein}$.

1.4.2. Experiment-based factors for estimation of immobilization quality

Further orientation on the catalytic efficiency of an immobilized enzyme can be given by relating the maximum catalytic rates to the K_m values of a given catalyst in fixed and immobilized form. The ratio V_{max}/K_m the catalytic efficiency of an enzyme-substrate pair:

$$catalytic\ efficiency = \frac{V_{max}/K_m(immob.)}{V_{max}/K_m\,(free)} \tag{1-18}$$

In order to estimate the activity yield upon immobilization a balance between the enzymatic input and output activity should be made up. This factor is set up of:

$$activity\ yield = \frac{absolute\ enzyme\ activity\ of\ bound\ enzyme\ (U)}{absolute\ enzyme\ activity\ prior\ to\ immobilization\ (U)} \cdot 100\ \% \tag{1-19}$$

The activity yield summarizes all effects of immobilization influencing the enzyme activity. Such effects include substrate transport limitations as well as conformational changes of the biocatalysts occurring upon immobilization. A particular distinction of single effects may not be made.

1.4.3. Selected examples for comparison of porous and non-porous biocatalyst carriers

The comparison of mass transport phenomena taking place in real biocatalyst systems links theoretical approaches and practical examples. The importance of substrate availability, particles size and porosity is case specific. Apart from mass transport considerations, additional factors affecting the efficient use of immobilized biocatalysts are carrier specific surface, long term stability, susceptibility to pH, temperature, oxygen and other substances. The subsequent part of the chapter deals with examples from recent research on the topic and compares actual porous and non-porous carrier systems. Here the focus is set on the comparison of estimated effectiveness factor η and experiment-based activity yields in mostly industrially applied enzymes. From the information given in the publications the theoretical effectiveness factor has been estimated. For these estimations the equations 1-3 to 1-8 and 1-12 to 1-16 from section 1.4.1 were used. In cases, where certain parameters needed for the estimations were missing, data from data bases and other publications were used.

1.4.3.1. Effects of the substrate properties on the effectiveness factor of porous and non-porous biocatalyst carriers

The overall reaction rate of a carrier immobilized enzyme is determined by enzyme- and carrier-related characteristics, and substrate properties prevailing in the bulk solution. Substrate properties-related factors such as concentration, solubility, diffusivity and chirality influence the mass transfer rate and thus the substrate availability to the enzyme molecule. These properties may have only minor affects to unbound enzymes but may be crucial factors for the overall effectiveness factor of bound enzymes. Thus, *a priori* to a final choice of a suitable carrier system the major influencing factors of substrate availability to the enzyme should be considered. The effects of the substrate properties on the effectiveness factor selected from literature are discussed in the following.

The effect of substrate solubility on the effectiveness factor of porous and non-porous enzyme carriers is discussed in the work of (Magario *et al.* 2008). Magario and coworkers used naringinase from *Penicillium decumbens* as a model enzyme for comparison of micro-magnetic non-porous particles with a diameter of 2 µm and porous carriers (Eupergit and Sepharose) 34-190 µm by

size. The cleavage of an α-L-rhamnose molecule of a dirhamnolipid is performed by naringinase. Under reaction conditions used in this work nearly total rhamnolipids are bound to micelles, whereas only truly dissolved molecules, with a stated diffusion coefficient within 10^{-10} m^2/s, are available for enzymatic hydrolysis. Thus, the substrate concentration is reported to be very low, and amounted to 0.05 mM at reaction conditions. For low substrate concentrations when the dimensionless substrate concentration β_b approaches 0 ($s_0 << K_m$) the substrate mass transport becomes the limiting overall reaction rate factor. Magario and coworkers reported that the highest immobilized activities were obtained by use of the non-porous micro-magnetic beads. These non-porous micro-magnetic carriers were reported to be the most appropriate carrier for bioconversion of a low-soluble substrate like rhamnolipid, where the influence of strong diffusion limitations of the substrate on the overall reaction rate in three phase reaction process is avoided in comparison to porous supports. However, the smaller specific surface available on non-porous particles limited the specific carrier activity obtained at high protein loadings. Successive reducing the particle size of non-porous micro-magnetic carriers could result in an improvement for this sort of application.

Further examples of effects of substrate solubility and diffusivity on the effectiveness factor of different enzyme immobilizates are described in the works of Dincer and Telefoncu (2007); Liao *et al.* (2008) and Liao *et al.* (2010) (Dincer and Telefoncu 2007; Liao *et al.* 2008; Liao *et al.* 2010). Liao and coworkers reported on the synthesis of a novel polyvinyl alcohol/Fe_2O_3 magnetic nano-particle under micro-emulsion system for the immobilization of cellulase with high activity. In this work the immobilized cellulase was described as a kind of spherical complex with a diameter of approximately 270 nm, and mainly constructed by the accumulation of 10 nm Fe_2O_3 nano-particles with the diffused polymer. A previously by Liao *et al.* (2008) published method for immobilization of cellulase in an aqueous solution system on PVA/Fe_2O_3 nano-particle (diameter of 1 µm) was compared to this method (Liao *et al.* 2008). Both immobilized cellulases were applied for degradation of microcrystalline cellulose. The reported activity yields for the immobilizates prepared under microemulsion or aqueous systems amounted to 91% and 63%, respectively. However, from the description given in these two reports it is unclear whether the cellulase is situated inside or on the

surface of the particles. Due to the low solubility and diffusivity of the substrate at given conditions a diffusion coefficient of $D_{eff} \approx 10^{-22}$ m²/s is estimated. Penetration of a microcrystalline substance particle to a carrier particle becomes improbable. The authors compared their results to the method reported by Dincer and Telefoncu (2007), where cellulase was immobilized on the surface of 820 µm maleic anhydride modified coated PVA with chitosan as form maker (Dincer and Telefoncu 2007). The researchers measured the activity of cellulase immobilizates by degradation of soluble carboxymethyl cellulose. The use of a surface immobilized enzyme carrier and a soluble substrate resulted in a reported activity yield of 87%. The estimated effectiveness factor value for the poorly soluble cellulose lies within 100% for non-porous immobilized enzyme while for porous supports it approaches 0%. Consequently comparing all three works it is more than likely that the enzyme immobilized in the works of Liao and coworkers (2008 and 2010) is mostly situated on the surface of the supports. In combination with the better reported activity yield of the enzyme carrier prepared in microemulsion systems the size of the applied particle seems to be a crucial factor for the immobilization of cellulose. Smaller particles offer a larger surface area to immobilization and allow for better contact with the actual substrate and are thus more appropriate in the case of a microcrystalline substrate. Working with higher soluble cellulose such as in case of Dincer and Telefoncu (2007) may increase the activity yield even for cellulose immobilized on the surface of larger particles with mean diameters of 820 µm.

Examples for the effect of the conversion of low molecular mass substrates such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and syringaldazine by immobilized laccase on porous and non-porous carriers is discussed by means of subsequent examples. Pich *et al.* (2006) used laccase immobilized on non-porous polymeric particles possessing a polystyrene core with maghemite and reactive β-diketone groups located on the particle surface acting as binding sites for the enzymes. These nano-particles have shown an activity yield of 20% using ABTS as substrate (Pich *et al.* 2006). Arica *et al.* (2009) reported on achieving better results in the activity yield by immobilizing laccase onto spacer-arm attached non-porous poly(glycidylmethacrylate/ethylene-glycoldimetacrylate) beads. The achieved activity yield for syringaldazine conversion was over 80% (Arica *et al.* 2009). Another work investigated laccase immobilized on porous particles. The

immobilization of laccase on meso-porous silica spheres gave activity yields of up to 80% depending on the immobilization method (Zhu *et al.* 2007). In these works ABTS and syringaldazine, as comparatively small model substrates, were used within $\beta_b > 10$ for non-porous particles (Pich *et al.* 2006) and $\beta_b > 1000$ for micro-sized porous particles (Zhu *et al.* 2007). As expected for relatively small and well soluble substrates the theoretical estimation of the effectiveness factors results in $\eta = 100\%$ for the mentioned systems, in which mass transfer limitation was not a relevant factor. Consequently both particle sorts are suitable for conversion of ABTS or syringaldazine. The relatively low activity yield observed by Pich *et al.* (2006), therefore, is most probably the result of other immobilization effects. However, practically the application of laccase ranges from low molecular weight substrates such as phenolic compounds, dyes and aromatic substrates to polycyclic aromatic hydrocarbon compounds and polyphenols with higher molecular weights. The conversion of the latter compounds presents a challenging task since the solubility and diffusivity of the substrate as well as the affinity of the enzyme to the substrate is drastically decreased compared to ABTS or syringaldazine. In this case the estimated effectiveness factor for non-porous supports would maintain a value of 98% while the effectiveness factor for porous supports would reach only about 40%. Correspondingly, the conversion of high molecular polyphenolic substrates would be more efficient with non-porous micro enzyme carriers.

The effect of mass transport resistances on the catalytic efficiency by using porous and non-porous micro-carriers in a model reaction as well as in preferably stereoselective reactions is discussed in the work of Bozhinova *et al.* (2004). Bozhinova and coworkers reported on experiments in which penicillin amidase was immobilized on non-porous polyvinyl alcohol, polymethylmetacrylate and polyvinylacetate divinylbenzene magnetic beads with particle size of 1.7-6 µm. The enzymatic activity was measured by spectrophotometric assay with the chromogenic substrate, 6-nitro-3-phenylacetamido benzoic acid with diffusion coefficient order of magnitude of approximately 10^{-9} m²/s. Penicillin amidase bound to larger and porous particles (Sepharose and Eupergit C, 80 and 150 µm, respectively) was reported to show apparent K_m values two orders of magnitude higher as compared to that of the free enzyme. For penicillin amidase immobilized on non-porous micro-beads, the apparent K_m was only 5-fold higher. As the catalytic efficiency is the ratio of $(V_{max}/K_{m(immob)})/(V_{max}/K_{m(free)})$ (equation 1-18)

both enzyme constants must be considered to assess the catalytic efficiency. Thus, considering the same reported order of magnitude of the turnover numbers for the compared immobilized enzymes, this implies higher specificity and catalytic efficiency for non-porous magnetic beads-bound enzyme. Apparently, the substrate concentration gradient on porous supports leads to a higher $K_{m,app}$ of immobilized biocatalysts due to higher mass transport resistance. Furthermore, additionally two different reactions catalyzed by porous and non-porous support immobilized penicillin amidase were investigated: a kinetically controlled enantioselective condensation reaction of *R*-phenylglycine with enantiomeric *S*- and *R*-Phe as well as equilibrium controlled enantioselective hydrolysis of a racemic substrate phenylacetyl-Phe. Bozhinova and coworkers stated a twice as high enantioselectivity during synthesis on the non-porous support compared to porous carriers. An even higher enantioselectivity of the non-porous support was found during the hydrolysis. Non-porous supports lead to a ten times higher enantioselectivity (Bozhinova *et al.* 2004). These results may be explained by an enantiomer effect in the solution surrounding the immobilized biocatalyst. Substrate selectivity decreases as the ratio of the enantiomers shifts in favor of the unpreferred substrate in the biocatalyst micro-environment. In porous supports the export of product is slower and the substrate import into the pores is more hindered in comparison to non-porous particles. Thus, it can be concluded that the usage of non-porous micro-particles for stereoselective reactions is more beneficial as compared to the usage of porous supports.

1.4.3.2. Effects of protein loading on effectiveness factor of porous and non-porous biocatalyst carriers

For reactions in which diffusion or the reaction rate is the major overall rate controlling step, the protein loading may be a helpful parameter to increase the effectiveness of porous as well as non-porous enzyme carriers. In the following section the influence of protein loading on effectiveness factor and activity yield by selected examples from literature is discussed.

In hydrolyzation reactions of high molecular mass substrates such as starch, the diffusion of the substrate to the immobilized enzyme may be an overall rate limiting factor. The influence of protein loading on the activity yield of porous enzyme carriers is discussed in the work of Siso *et al.* (1990). Porous siloxane and corn grits served Siso and coworkers for α-amylase

immobilization in starch hydrolyzation. The authors stated that surface immobilized α-amylase demonstrated a strong diffusion limitation of the substrate and product which was reflected in activity yields with highest value of 24% for corn grits and maximum value of 19% for siloxane. The observed decrease in activity yields were attributed to be related to the increase of enzyme loading. With protein loading of 7.3 mg/$g_{carrier}$ the highest activity yield of 24% was achieved, while with 100 mg/$g_{carrier}$ only 3.6% were achieved. The authors concluded that when enzymes are too tightly packed on the support, the rate of substrate conversion can exceed the diffusion rate of the substrate into the carrier matrix. With macromolecular substrates like starch such effects gain more relevance and the overall reaction rate becomes diffusion controlled (Siso *et al.* 1990). A possible improvement of activity yield may result from using non-porous supports, where diffusion limitation influence is lowered when the enzyme is situated only on the surface of the carrier.

Another example for the influence of the protein loading on activity yields of non-porous enzyme carriers in reactions with high molecular mass substrates is discussed by means of the work of Oh and Kim (2000). Oh and Kim used non-porous polystyrene/polysodium styrene sulfonate microspheres with mean size of 258 nm for immobilization of amyloglucosidase. Dextrin served as a model substrate from which glucose monomers were released by enzymatic digestion. It is reported that the Michaelis-Menten parameters, K_m and V_{max} were affected by the enzyme loading. The K_m of amyloglucosidase increased in relation to the enzyme loading whereas V_{max} decreased significantly. The authors stated that with higher enzyme loading of immobilized enzyme the microspheres began to agglomerate, and the diffusion limitations of substrate and product were induced. The highest activity yield of almost 90% was reached by the lowest enzyme loading of 7.1 mg/$g_{support}$. For these particles a catalytic efficiency of about 53% could be calculated from given K_m and V_{max} (Oh and Kim 2000). The authors concluded that the decrease in activity yield of these micro-particles was probably due to a large protein overload causing enzyme multilayer, where diffusion limitations show higher significance. Furthermore, the increase in K_m was explained by the authors by conformational changes of the enzyme bound to the carrier surface. However, the stated decrease in activity yield with increasing protein loading as well as the shift of the K_m could be also attributed only to diffusion limitation of the

substrate in relation to increasing reaction rate with higher amounts of immobilized enzyme on the carrier.

Examples for protein loading effects in water-in-oil emulsion surrounding pore-bound lipase are discussed by means of the works of Wu *et al.* (2009) and Gao *et al.* (2009). A lipase from *Candida rugosa* was immobilized on porous magnetic chitosan particles with sizes of 50-100 nm (Wu *et al.* 2009). Gao and coworkers accomplished an adsorptive immobilization of the same lipase on silica aerogels with particle sizes from 50 to 250 µm (Gao *et al.* 2009). In these publications the activity measurement was based on the conversion of olive oil with an estimated diffusion coefficient D_{eff} of 10^{-9} m²/s. Wu and Gao *et al.* reported that with increase of enzyme loading on the particles or of the free enzyme concentration in the media the activity of both free and immobilized lipase decreased. This decrease in immobilizate's activity was explained by the fact that with increasing enzyme loading from 0.5 to 5.5 mg, multiple layers of enzymes filled the pores of the named particles. Thus, the surface layer enzymes prevented contact between the substrate and enzymes in lower layers. The decrease of activity of the free enzyme was explained by hydrophobic interaction that caused the lipase molecules to aggregate and led to multilayer formation. Hence a decrease of the specific activity of the bound and free enzyme by 70% within increasing protein concentration is reported. However, another possible explanation for this decrease of the specific activity may be a mass transfer limitation effect within the pores of the enzyme carrier as well as within the free enzyme aggregates.

In other publications it was shown how the assessment of the mass transport effects on the conversion with immobilized acid urease may help improving the activity yield. In the works of Bortone *et al.* (2012 and 2013) examples are given where the mass transport limitation was investigated and lowered by shifting the operating range of the Thiele modulus. Immobilization of acid urease on porous Eupergit C with average particle size of 175 µm supports was conducted. The enzymatic activity was assayed by degradation of urea (with D_{eff} given as $1.29 \cdot 10^{-9}$ m²/s at reaction conditions). The influence of enzyme loading and substrate concentration on the effectiveness factor considering external and internal mass transfer limitation as well as only intraparticle diffusion (given as η) was theoretically assessed. By estimating the Weisz number W, the researchers calculated, that the activity of the biocatalyst with the highest enzyme loading at the lowest urea concentrations

was reduced by a factor of about 2 compared to the free enzyme due to diffusion limitations within the pores. This conclusion was further reinforced by the researchers on the basis of increasing K_m values of the immobilized urease with increasing protein loading. By operating in practical values of urea concentration occurring in wines with $s_0 << K_m$ ($\beta_b \rightarrow 0$) with immobilizates at lower immobilized protein concentrations their activity after grinding from 180 µm to a particle size of 5 µm was no more limited by intraparticle diffusion and approached the value of the free enzyme with effectiveness factor given as η = 100% (Bortone *et al.* 2012; Bortone *et al.* 2013). By adjusting the protein loading the effect of mass transport limitation could be diminished in this sort of reaction reaching the maximum effectiveness.

1.4.3.3. Effects of porous and non-porous carriers on the effectiveness factor

Besides enzyme and substrate related properties the effectiveness factor of a given reaction system is influenced by surface properties of the carrier and therefore contributes to the choice of a suitable carrier. Selected examples from literature in which the pore size or surface property of a carrier had a major influence on the effectiveness are discussed in the following.

Pore size dependant substrate accessibility to immobilized amyloglucosidase on different macro-porous supports was reported to be a crucial factor in the work of (Emneus and Gorton 1993). Amyloglucosidase immobilization has been performed by the use of three different particle types featuring four different pore sizes. The sizes of the used porous supports controlled pore glass, ceramic silica support and alumina-based carrier were 37-74 µm, 7-20 µm and 8 µm, respectively. Digestion of glycogen as highly branched starch-type polymer with low diffusivity of 10^{-20} m^2/s served as model reaction for activity determination. A thorough discussion on the relationship between reaction rates of amyloglucosidase for high molecular mass substrates and pore size, surface area and immobilization efficiency was presented. For controlled pore glass beads with the smallest pore sizes of 17 nm exclusion of the substrate from the pores occurred. This resulted in the lowest $K_{m,app}$ compared to carriers with larger pore sizes, indicating that only the surface immobilized and not the pore bound enzymes catalyzed the reaction on this carrier and the reaction was thus reported to be kinetically controlled. For the porous ceramic silica support and alumina-based carrier with larger pore

diameters (30 and 25 nm pore diameters; respectively) the substrate access to the pores and the reaction rate was reported to be controlled by diffusion which resulted in much lower apparent conversion rates compared to a controlled pore glass with pore size of 149 nm. The authors concluded that apparently substrate accessibility of the 25, 30 and 149 nm pores was not adequate where most of the enzyme was situated (Emneus and Gorton 1993). A support with a sufficiently large pore size or a non-porous particle would be necessary to prevent total exclusion of high molecular weight substrates such as glycogen and probably also native starch.

The effect of enzyme location on the carrier on the activity of trypsin and urease immobilized non-covalently on porous membranes by using layer-by-layer self-assembly of a polyelectrolyte has been investigated by Guedidi *et al.* (2010). The studied systems consisted of bilayered assemblies with the enzyme layer as the outer layer and trilayered assemblies with the enzyme layer as the inner sandwiched layer. The activity of trypsin was determined by cleavage of *N*α-benzoyl-L-arginine-4-nitroanilide, while urease was used for hydrolyzation of urea. Trypsin hidden in the pores of the polyacrylonitrile based membrane showed the lowest specific activity, while the more exposed form of the enzyme on the outer layer revealed a factor 1.5 higher specific activity. For urease located inside the pores of the polymer as well as covered with a layer of polyethyleneimine the lowest activities were observed. Urease deposited as the outer layer of the assembly showed the best activity results. Although polyelectrolyte covering the enzyme layer appears to provide a better environment to ensure long-term enzyme stability, the absolute specific activity of immobilized enzymes was found to be about 1-10% of that of the free ones in solution (Guedidi *et al.* 2010). In this work the effects of mass transport limitation on the enzymes situated in the inner layer and covered by a porous outer layer are demonstrated. The porous nature of the outer layer prevented the appropriate penetration of the substrate leading to mass transfer limitations which were responsible for overall reaction rate decrease.

Kalantari *et al.* (2012) investigated three types of magnetic supports for lipase immobilization. One non-porous silica carrier with particle size of 300 nm was compared to two types of meso-porous silica particles 450 and 500 nm by size, respectively with small and large pore sizes. The conversion of p-nitrophenol palmitate (pNPP) with estimated D_{eff} of 10^{-6} m^2/s was used for activity measurements. The results showed that the immobilization of the lipase led to

a significant decrease in the enzyme's affinity to the substrate. The K_m values for the immobilized lipase on all tested particles were 1.8 to 2.2 times higher compared to the free enzyme which indicates a conformational change in enzyme structure upon immobilization. The catalytic efficiency of lipase on non-porous supports was reported to be 51% and exceeded the catalytic efficiencies of small and large porous supports showing 44% and 41% catalytic efficiencies, respectively. Furthermore the activity yields for both porous supports resulted in 87% and were lower compared to the non-porous support where the yield amounted to 91% (Kalantari *et al.* 2012). The estimated expected effectiveness factor lies within 100% under given conditions for all three particle sorts. The results of Kalantari *et al.* (2012) indicate that non-porous particles were more suitable for higher activity yields than porous lipase carrier. An assumption can be made, that the immobilization technique contributes to the lower activity yield compared to the expected effectiveness factor for all three particle sorts.

1.4.3.4. Effects not covered by mass transfer transport to the carrier model

Enzyme immobilization offers a useful technique allowing reutilization of costly biocatalysts. Micro non-porous carriers feature reaction rates which underlie less mass transport limitation effects, while porous supports often display a higher specific surface for protein immobilization. On the other hand a number of non-mass transfer related phenomena reveal some disadvantages of carrier application at operational optimum and need to be overcome. Such factors are:

- enzymatic denaturation
- unfavorable conditioning of the immobilization method
- carrier micro-environment of reaction solution
- steric hindrance of the enzymes in vicinity of each other
- protein multilayer formation

The effects which are not covered by the assessment of the effectiveness factor of the carrier will be discussed in the following by means of selected literature.

The adverse effect of reaction media in a stirred system on the effectiveness factor of porous and non-porous carriers is discussed by following example. An immobilization of glucose oxidase (GOX) was investigated by Betancor et al. (2005) who compared agarose binding (particle size 45-165 µm) and non-

porous magnetic support binding (particle size 0.8 µm) as well as subsequent coating of the enzyme by dextran aldehyde. The enzyme activity was determined by conversion of the model substrate ABTS. The diffusion coefficient D_{eff} for this substrate lies within 10^{-10} m^2/s. Estimating the effectiveness factors η from the given data for both porous and non-porous particle species showed an overall reaction rate which is not affected by mass transport limitation with η =100%. Hence both particle sorts are suitable for this kind of reaction. However, the authors stated that the enzyme immobilized on nano-particles was inactivated in a very similar fashion to that of the soluble enzyme in a vigorously stirred system. This implies that the mere immobilization of the enzyme on this non-porous support did not prevent the enzyme inactivation by the presence of gas bubbles. Apparently the protection of the enzyme by dextran aldehyde decreases the inactivation by gas bubbles in a stirred system (Betancor *et al.* 2005).

The cleft between the theoretical evaluation of the mass transfer effect within an immobilization technique and the practical value of the activity yield is discussed by following example. Immobilization of jack bean urease on non-porous spacer-arm incorporated micro-beads with mean particle size of 115 µm was investigated by Ayhan *et al.* (2002). Urea was used for enzymatic activity measurement. The diffusivity of the comparably small substrate lies within 10^{-9} m^2/s The rate-controlling step in urea conversion was determined by the researchers by accounting the Weisz number (observable Thiele modulus Φ_{obs} in this publication), which was less than 0.3. Thereby the rate-controlling step was found to be the enzymatic reaction. Despite the low mass transport limitation the specific activity suffered from drastic decrease (activity yield of only 0.03%) which was attributed to steric effects, conformational changes and denaturation of the enzyme during the immobilization process (Ayhan *et al.* 2002). Consequently although mass transport limitation is less significant in the stated reaction, the immobilization method led to lowered activity yields. Thus, despite an assessment of mass transfer effects was accomplished in the stated publication, in this reaction system probably the immobilization protocol caused conformational changes of the enzyme and crossed high activity yields.

In general, some suggestions to avoid the in section 1.4.3 discussed drawbacks are offered in the following. The stated problem of limited specific area on non-porous carriers may be solved by successive minimizing the

particle size. Occurring multilayer formations may be avoided by optimizing the protein loading. A more profound approach provides the molecular understanding of the effects appearing upon enzyme immobilization such as enzyme orientation on the carrier, micro-environment changes, enzyme-carrier-interactions and the influence of their mutual properties as well as the particle mobility itself (Abian *et al.* 2001; Jia *et al.* 2003; Mateo *et al.* 2007; Montes *et al.* 2007; Gleeson *et al.* 2011).

It is evident that mass transport limitations affect many enzymatically catalyzed reactions in heterogeneous systems. The selected examples presented here show that an a priori estimation of the effectiveness factor η of the reaction system may be advantageous before selecting carrier materials for laboratory testing.

Micro-magnetic non-porous carriers show the potential for advantageous applications for diverse biocatalytic systems even up to the industrial scale. Such micro sized particles are suitable for processes where the substrate is hardly available due to low solubility, crystal or complex formation and also in case of substrates with high molecular weights where diffusion to the biocatalyst is very limited.

The application of non-porous micro magnetic particles in biomedical laboratories is very common for separation purposes. In contrast, the application of magnetic micro- and nano non-porous carrier in enzymatic reactions is still unusual at present and the industrial use being a vision for the future.

1.5. Research proposal

The application of purified enzymes for industrial biotransformation processes affords ways to separate and reuse the enzyme to lower the costs of the process. However, the methods of enzyme immobilization are diverse and researchers are confronted with the need of an individual immobilization method depending on the enzyme and its application in the reaction process. This results in a magnitude of immobilization methods for each individual enzyme. The immobilization method may provide the biocatalyst with altered qualities such as activity, specificity, stability, optimum conditions or mass transfer behavior. Trypsin is an industrially significant biocatalyst applied for peptide hydrolysis and synthesis reactions in different organic and aqueous media. However, in examples from literature the immobilization of trypsin often led to activity decrease. Moreover, most research publications dealing with trypsin immobilization test the tryptic activity for a standard photometric hydrolysis activity assay. Such activity measurement may not provide sufficient information about enzyme's properties after immobilization. The insights from this method may be badly transferable on actual industrial biotransformations with trypsin. Furthermore, mostly only one immobilization method is investigated in one publication in order to improve the enzymatic activity upon immobilization resulting in poor comparability of different immobilization approaches.

This study should seize the listed set of issues connected with immobilization approaches for trypsin. A contemplation of immobilization efficiency should be approximated from three main directions. These should comprise immobilization methods and physical and chemical material characteristics that are shown to be most significant influence factors. The immobilization medium should be engineered and particle surface and size should be evaluated. The evaluation of the altered immobilization characteristics should be based on a reference immobilization method from literature and on comparison with free trypsin. Response factors should be defined to estimate the impact of each influence factor and method. These response factors should comprise enzymatic activity, specificity, reusability, mass transport and by-product formation. From this contemplation a more holistic approach to enzyme immobilization method development should be achieved.

In order to transfer the compiled immobilization methods onto industrially relevant reactions part of this study should be development of model activity

assays. A peptide synthesis assay in organic medium should be set up as well as a standard hydrolysis assay used in research publications. The latter provides for a better comparability to previous works. Finally, to support the immobilization method evaluation, by-product formation in a peptide synthesis/hydrolysis system should be investigated. A special model reaction of trypsin-catalyzed peptide oligomer formation should be evolved as a test system.

The gained perceptions from listed investigations should serve to evaluate enzymatic behavior in diverse applications.

The outline of this work is given as a scheme in Figure 1.15.

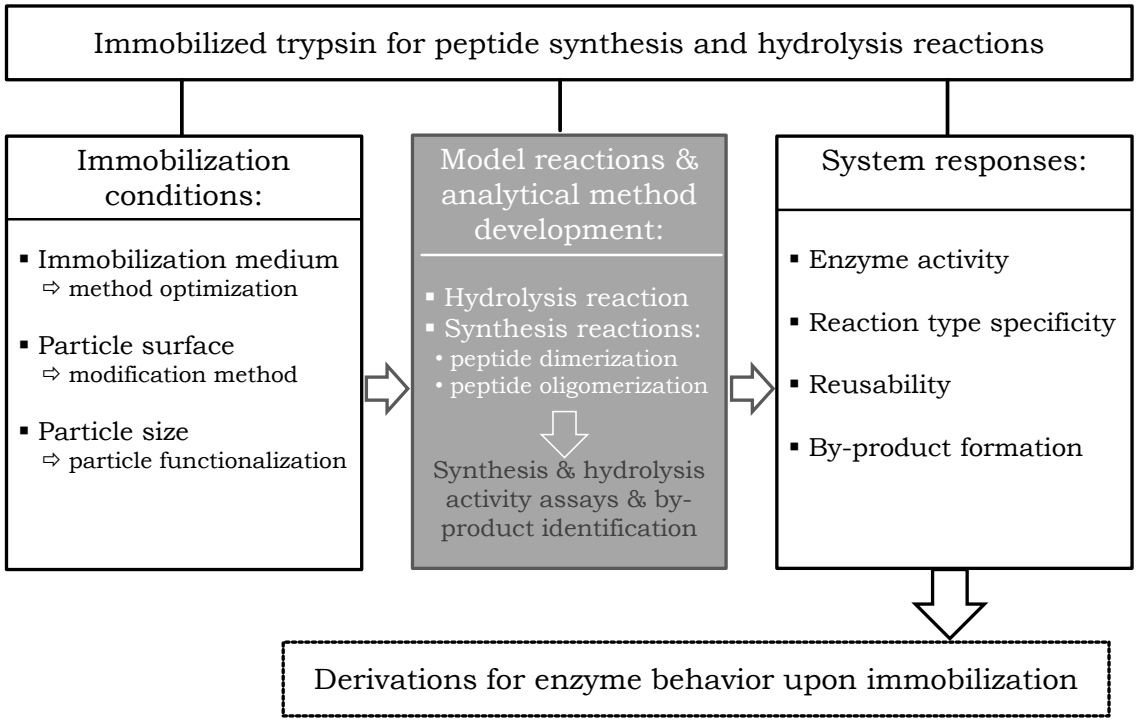


Figure 1.15. Graphical outline of the present work.

2. Evaluation of immobilization method

This chapter is partially based on the publication

IMMOBILIZATION OF TRYPSIN IN ORGANIC AND AQUEOUS MEDIA FOR ENZYMATIC PEPTIDE SYNTHESIS AND HYDROLYSIS REACTIONS

Julia Stolarow[a], Manuel Heinzelmann[a], Wladimir Yeremchuk[a], Christoph Syldatk[a], Rudolf Hausmann[b]

[a]Institute of Process Engineering in Life Science, Section II: Technical Biology, Karlsruhe Institute of Technology, Karlsruhe, Germany [b]Department of Bioprocess Engineering, Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

---

The final publication is available at http://www.biomedcentral.com/

Bibliographic details:

Published in *BMC Biotechnology* 15:77, August 19, 2015, pp 1-8
Springer Open

DOI: 10.1186/s12896-015-0196-y

Authors' contributions to the publication:

Julia Stolarow carried out the major experimental work, conceived, and wrote the manuscript.

Manuel Heinzelmann carried out the hydrolytic activity measurements

Wladimir Yeremchuk contributed to synthetic activity assay setup.

Christoph Syldatk designed the project, supervised the work, and provided administrative support.

Rudolf Hausmann contributed to the design of the study, interpretation of data, and revised the manuscript.

2.1. Using enzymes in micro-aqueous media

Micro-aqueous organic media have beneficial effects on enzymatic stability in synthesis reactions where the enzymes may become more rigid and thus less susceptible to conformational changes (Klibanov 2001). Proteins have been reported to show a "pH-memory" of the last aqueous medium prior to lyophilization and are able to maintain their ionization state in micro-aqueous organic solvents (Zaks and Klibanov 1985; Costantino et al. 1997). When lyophilized from a pH corresponding to the optimum pH, the activity of the lyophilized and organic solvent resubstituted enzyme has been described to be at its maximum (Zaks and Klibanov 1985). These effects have been investigated among different enzymes catalyzing synthesis reactions in organic media (Zaks and Klibanov 1988; Yang et al. 1993; Sergeeva et al. 1997). The knowledge of these effects may act as a useful tool for immobilization of enzymes.

The application of organic solvent systems for immobilization of hydrolases is so far limited to few publications. One of the first investigations on organic solvent immobilized lipase was done by Stark and Holmberg (1989). The researchers compared aqueous, microemulsion and organic systems for immobilization to test the lipase activity in hydrolysis and transesterification reactions. Within transesterification reaction the organic and microemulsion systems showed 20% and 40% residual activities, respectively, for immobilization while within aqueous method no transesterification activity after immobilization could be detected (Stark and Holmberg 1989). In the works of de Castro *et al.* (1999) and Soares *et al.* (1999) lipase from *Candida rugosa* was immobilized in apolar and polar solvents such as hexane and acetone and compared to aqueous immobilization in buffer. The results showed a sevenfold higher specific activity of acetone immobilized lipase in butyl butyrate synthesis reaction compared to aqueous solution immobilization (de Castro *et al.* 1999; Soares *et al.* 1999). Sun *et al.* (2010) immobilized lipase from *Candida antarctica* by adsorption in isooctane. This immobilization achieved an up to threefold higher activity recovery compared to adsorption in aqueous medium (Sun et al. 2010). Zhu *et al.* immobilized several enzymes including trypsin within micro-aqueous organic media onto chitosan microspheres. Immobilization under micro-aqueous conditions showed a twofold higher remaining catalytic activity compared to water phase immobilization (Zhu *et al.* 2011). These results appear to show a promising

alternative to the mostly used aqueous immobilizations. Thus a method development for the model enzyme and particles used in this work is required.

2.2. Experimental design approach

Method optimization may be conducted by different strategies. First significant influence and response factors must be defined. Subsequently, a variation of influence factors gives information about how responses change in dependence of each influence factor. Variation of influence factors one-by-one leads to response factor models consisting of one factor, but no factor interaction may be observed. In a statistical experiment design approach a poly-dimensional space is created showing the influence and interference of different influence factors on one specific response (Dejaegher and Vander Heyden 2011). In a response surface design two factor levels for each influence factor are determined representing high and low factor values. Furthermore, a center point representing an intersection of factorial points and star points representing parameters with a distance of α to the center point are computed (Figure 2.1). In previous works statistical design approach was used for optimization of immobilization methods (Mukherjee *et al.* 2010; Li *et al.* 2013). Immobilization influence factors such as amount of particles, immobilization pH, reaction temperature and amount of enzyme were varied.

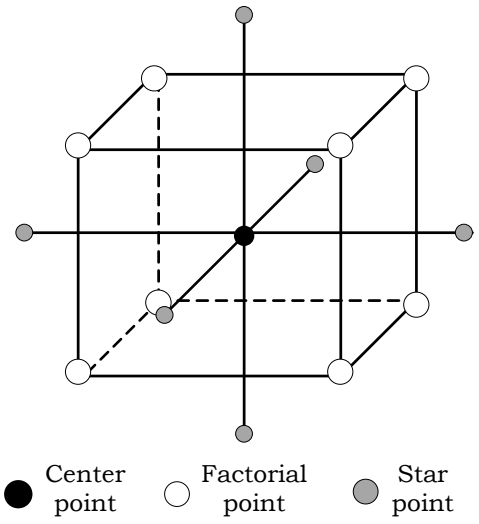


Figure 2.1. Experimental space of a response surface design. Each point depicts an experiment with a certain influence factor combination.

The intention of the work presented in this chapter was to elaborate and evaluate a method for immobilization of trypsin in organic medium which improves the remaining activity upon immobilization compared to a more common immobilization technique in aqueous medium. A special focus was

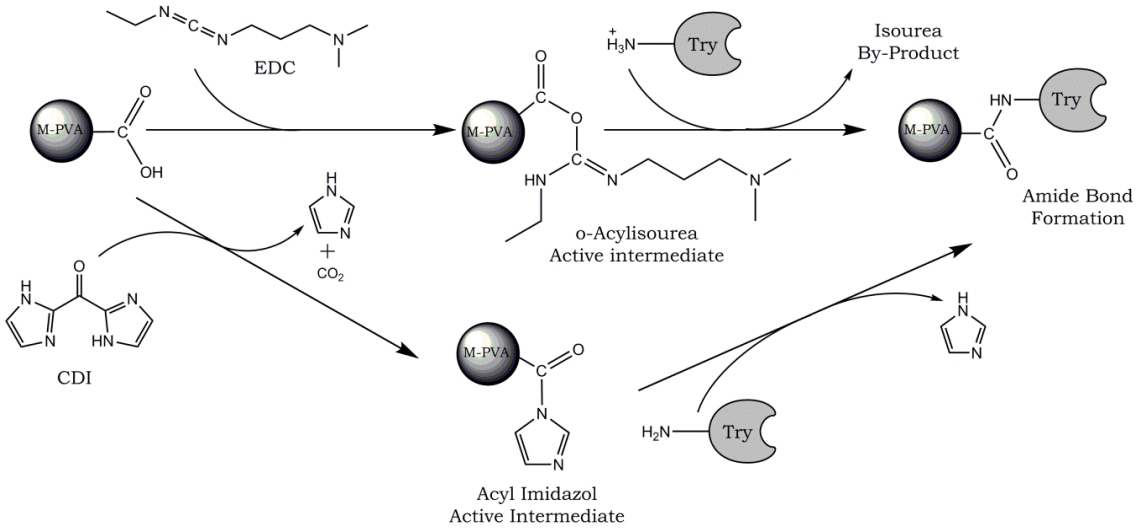
set on the comparison of these immobilization methods in both possible trypsin-catalyzed reaction types: a standard-substrate hydrolysis reaction which is performed at physiological conditions in aqueous medium as well as synthesis reaction in organic medium, which plays an important role in industrial drug production.

2.3. Materials and Methods

Magnetic poly(vinyl alcohol) micro-particles (M-PVA) C22–C250 were provided by PerkinElmer Chemagen, Baesweiler, Germany. The used beads feature a carboxyl functionalization with a concentration of 950 µmol $COOH/g_{particle}$ and particle size distribution within 1-3 µm. 1,4-dioxane, 1-propanol, methanol, ethanol, acetone, acetonitrile, 2-(N-morpholino)ethanesulfonic acid (MES), carbonyldiimidazol (CDI), calcium chloride, tris(hydroxymethyl)aminomethane (Tris) and trifluoroacetic acid (TFA) were purchased from Carl Roth, Karlsruhe, Germany. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), Nα-benzoyl-L-arginine ethyl ester hydrochloride (Bz-Arg-OEt), Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA), p-nitroaniline (pNA), L-arginine amide dihydrochloride (Arg-NH_2), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) and 4-(cyclohexylamino)-1-butanesulfonic acid (CABS) were purchased from Sigma-Aldrich, Deisenhofen, Germany. Recombinant porcine trypsin (EC 3.4.21.4) was purchased from Roche Diagnostics, Mannheim, Germany. Nα-Benzoyl-arginyl-arginine amide trifluoroacetate (Bz-Arg-Arg-NH_2) was synthesized by Bachem, Weil am Rhein, Germany. Nα-Benzoyl-L-arginine (Bz-Arg) was obtained from ABCR GmbH, Karlsruhe, Germany. BC Assay was performed by a BC Assay Protein Quantitation Kit from Interchim, Uptima, Montluçon, France. HPLC analysis was performed with a standard HPLC device from Agilent 1200 Series, Agilent, Waldbronn, Germany equipped with a reversed phase column (C18 Luna, 5 µm, 250x4.60 mm, Phenomenex, California, USA).

2.3.1. Covalent immobilization of trypsin in organic and aqueous media

For immobilization in organic media the protocol was as follows. The M-PVA particle suspension concentration was adjusted to 25 mg/ml and washed with 0.1 M MES buffer pH 5.3 for three times followed by four washes in dried 1,4-



Scheme 2.1. Experimental procedure of trypsin immobilization in buffered solution via EDC and in organic solvent via CDI.

dioxane with >99.8% purity. For activation of functional COOH-groups on particle surface CDI was used as activating substance at 0.12 $g_{CDI}/g_{particle}$ and incubated with particles at 18 °C for 82 min. After activation particles were washed with 1,4-dioxane for two times. Trypsin was lyophilized prior to immobilization, suspended in dioxane with given water content from 0-10% at concentrations of 0.002-0.080 $g/g_{particle}$ and incubated at RT for 1 h.

For immobilization in aqueous media a modified protocol according to (Hermanson 2008) was applied. The pH 5.3 adjusted 25 mg/ml particle suspension was activated by a 1.6 $g_{EDC}/g_{particle}$ EDC solution for 6 min at 11 °C and washed two times with 0.1 M MES buffer pH 7. Trypsin solutions in a range of 0.002-0.080 $g/g_{particle}$ in 0.1 M MES pH 7 were added to the particles and incubated for 30 min at 25 °C for enzyme coupling.

After enzyme coupling step the particles from each immobilization method were washed with 0.04 M Tris buffer pH 9 for three times and stored in 0.02 M $CaCl_2$ solution at 4 °C. The coupling supernatant and washing solutions were used for quantification of the bound enzyme by BC Assay. The particle concentration after immobilization was determined gravimetrically by drying 250 µl of the respective immobilizate at 70 °C for at least 16 h in micro-tubes. The procedure for both immobilization methods is shown in Scheme 2.1.

2.3.2. Synthetic and hydrolytic activity measurement of free and immobilized trypsin

For measurement of synthetic trypsin activity 200 µl of a 25 mg/ml immobilizate suspension were used removing the supernatant by

magnetization. 1.5 ml of a substrate solution containing 10 mM Bz-Arg-OEt, 200 mM Arg-NH_2 in 80% (v/v) ethanol and 20% 0.1 M Tris buffer pH 9 with 0.02 M $CaCl_2$ was added to the immobilizates starting the synthesis reaction. The reaction was conducted in 2 ml plastic tubes at 20 °C and 1400 rpm in an Eppendorf thermomixer. Reaction mixture samples were taken during linear product formation phase in different time intervals without particle uptake and mixed in a 1:4-ratio with 6% (v/v) TFA in order to terminate the reaction. For measurement of free trypsin, an enzyme concentration of 0.1 mg/ml in 1.5 ml substrate solution was used. Samples were analyzed by HPLC. 1 Unit of synthetic trypsin activity is defined as 1 µmol of synthesized Bz-Arg-Arg-NH_2/min of reaction.

Hydrolytic activity determination was accomplished using a 1 mM BAPNA solution in 0.1 M Tris buffer pH 8 containing 0.02 M $CaCl_2$. The reaction was conducted at 30 °C and initiated by introduction of 1.5 ml of substrate solution to 200 µl particles without supernatant. The reaction was conducted in 2 ml plastic tubes at 1400 rpm in an Eppendorf thermomixer. Samples were taken and the reaction was terminated by a 1:4-ratio of sample to 6% TFA solution. For determination of free enzyme activity a solution of 0.01 $mg_{trypsin}$/ml in 1.5 ml of substrate was used. Samples and pNA standard were analyzed in a microplate reader at 405 nm. 1 Unit of hydrolytic trypsin activity is defined as 1 µmol of pNA released per minute of hydrolysis reaction.

2.3.3. Protein quantitation assay

Protein quantitation of protein stock solutions and supernatants was performed using a standard BC Assay Protein Quantitation Kit. The assay was used in order to quantify the protein loading of immobilized enzyme measured against bovine serum albumin standard in the respective buffer solution.

2.3.4. High pressure liquid chromatography (HPLC) analysis of peptide synthesis samples

Peptide synthesis samples were analyzed by RP-HPLC-UV/Vis in a 18%/82% ACN/H_2O solution as mobile phase containing 0.05% TFA, at a column temperature of 60 °C for 13 min at 254 nm wavelength. As standard substances Bz-Arg-OEt, Bz-Arg and Bz-Arg-Arg-NH_2 were used in order to quantify the product concentrations.

Response parameters giving the protein binding and activity binding parameters (equations 2-1 to 2-5)for different immobilization methods were calculated after product quantitation in each individual assay.

$$\text{protein coupling yield} = 100\% \times \frac{\text{exp. protein loading } [g_{\text{bound protein}}/g_{\text{particle}}]}{\text{theor. max protein loading } [g_{\text{protein input}}/g_{\text{particle}}]}. \quad (2\text{-}1)$$

$$\text{specific carrier activity} = \frac{U}{g_{\text{particle}}}. \quad (2\text{-}2)$$

$$\text{activity coupling yield} = 100\% \cdot \frac{\text{exp. specific carrier activity [U]}}{\text{theor. specific carrier activity free enzyme [U]}}. \quad (2\text{-}3)$$

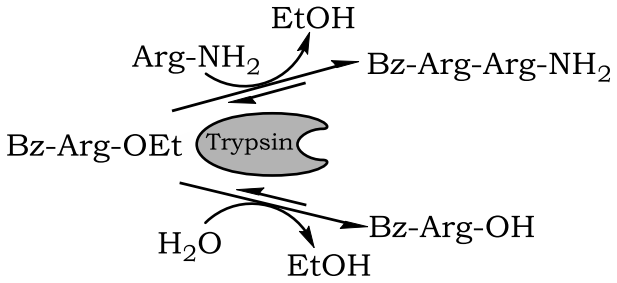
$$\text{specific activity} = \frac{U}{g_{\text{protein}}}. \quad (2\text{-}4)$$

$$\text{relative specific activity} = 100\% \times \frac{\text{exp. specific activity } [U/g_{\text{protein}}]}{\text{specific activity free enzyme } [U/g_{\text{protein}}]}. \quad (2\text{-}5)$$

2.4. Results and Discussion

2.4.1. Development of enzymatic synthesis of Bz-Arg-Arg-NH_2

For the synthesis of Bz-Arg-Arg-NH_2, which may act as an intermediate of several bioactive peptides, a kinetically controlled synthesis approach was selected (Ahmad *et al.* 1995; Kastin 2013). The reaction overview is given below. Bz-Arg-OEt was used as C-terminus activated and N-protected substrate. Trypsin binds Bz-Arg-OEt under release of ethanol forming an acyl intermediate which can be attacked by a nucleophile such as an amino containing component or water. In this reaction Arg-NH_2 acts as a nucleophile forming a peptide bond where the dipeptide Bz-Arg-Arg-NH_2 is released (see reaction Scheme 2.2). Formation of dipeptide in this reaction was confirmed by MALDI-ToF/MS analysis with an expected molecular mass of [M+H]+= 434.26 Da and a found molecular mass of 433.73 ±0.5 Da (see Figure A.1 of supplemental material). Figure A.2 of supplemental material gives the HPLC calibration results of Bz-Arg-Arg-NH_2.



Scheme 2.2. Trypsin-catalyzed conversion of Bz-Arg-OEt to a dipeptide amide with Arg-NH_2 and hydrolysis reactions.

An optimization of reaction parameters for synthesis reaction type was conducted. Reaction temperature, pH, water content and molar ratio of reactants were varied. Figure 2.2 shows the effects of different parameters on specific activity of free trypsin. The optimal water content for this reaction lies at 20%. This value is in accordance with previous works (Pugniere *et al.* 1986; Zhou *et al.* 2011). Optimal reaction temperature is 20°C. The optimum molar ratio of Bz-Arg-OEt to Arg-NH_2 amounts to a molar excess of the nucleophile Arg-NH_2 of 1:20. The optimum pH for this reaction is shifted to alkaline conditions between pH 8-9 with Tris buffer, pH 10 with CAPSO buffer and pH 11 with CABS buffer. For conducting further synthesis activity assays following standard conditions were applied: 20 °C, 20% (v/v) of water in ethanol, 20-fold molar excess of Arg-NH_2 and Tris buffered pH 9 of aqueous reaction medium moiety in ethanol.

2.4.2. Screening for suitable solvent for enzyme immobilization

For optimization of trypsin immobilization in organic medium a screening for the best suitable organic solvent was accomplished. A constant protein input of 0.025 $g_{trypsin}/g_{particle}$ was used. The results showed dioxane as best suitable medium synthesis carrier activity of trypsin upon immobilization (Table 2.1). However, no correlation to the solvent properties was found.

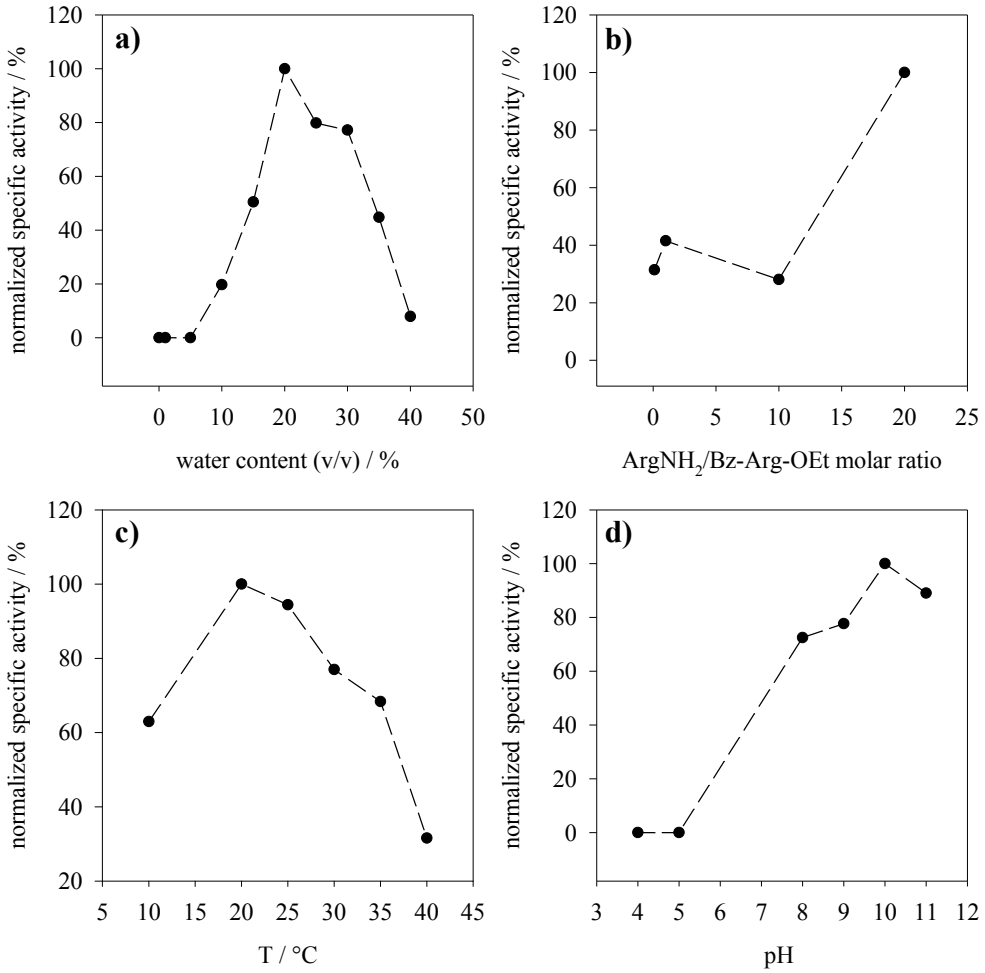


Figure 2.2. Biochemical characterization of Bz-Arg-OEt and Arg-NH_2 coupling reaction conditions: a) water content in medium, b) molar ratio, c) temperature and d) pH optimum.

Table 2.1. Relative carrier activity for immobilized trypsin in synthesis assay in different organic solvents with various physical properties.

	Relative carrier activity / %	Log K_{OW}[a]	Relative polarity[b]
Methanol	-	-0.74	0.762
Ethanol	-	-0.3	0.654
Acetone	45.1	-0.24	0.355
1,4-Dioxane	100.0	-0.27	0.164
1-Propanol	81.2	0.25	0.617

- no activity could be detected within this method. [a] log K_{OW} values were extracted from GESTIS data base of chemicals. [b] relative polarity was taken from (Reichardt 2004).

2.4.3. Immobilization method optimization by statistical design approach

For further optimization of organic immobilization method in dioxane, three influence factors were tested: concentration of the activating agent CDI, activation and coupling time. The optimization was conducted by statistical experiment design approach. This approach allows for identification of parameter interactions and faster optimization results. Response parameters such as protein coupling yield, activity coupling yield, relative specific activity and reusability were defined. 31 experiments were conducted at coded factor combinations in response surface method. Activator concentration was varied between 3 and 62 mg/ml representing 0.12-2.5 $g_{CDI}/g_{particle}$, activation time was varied between 6 and 149 min, coupling time was varied between 1 and 6 h. Table A.1 of supplemental material gives the factor combinations for each experimental run. Table 2.2 contains resulting models and model characteristics computed for the response parameters. All resulting models for response factors were significant. Factor interaction was found for protein coupling yield, relative specific activity and reusability. The experiments showed a minor influence of CDI concentration in the tested range. The lowest tested amount of CDI of 0.12 $g_{CDI}/g_{particle}$ results in 740 $\mu mol_{CDI}/g_{particle}$ and may activate approximately 78% of the functional COOH-groups on magnetic particle surface. However, the functional group density of a magnetic particle exceeds the available particle surface for trypsin coupling in a monolayer providing that a lower amount of activated COOH-groups is sufficient. R^2 values of all models are significantly low indicating further factors are required in order to depict the response behavior.

Figure 2.3 summarizes the influence of significant factors on all response parameters. The increasing of activation time had a positive effect on activity coupling yield and relative specific activity, but a decrease in protein coupling yield. Further, an increase in coupling time in the tested range led to increasing relative specific activity, but decreasing protein coupling yield, activity coupling yield and reusability. Target values for response factors were set. Constraints leading to calculated optimum influence factor combination are given in Table A.2. The final optimum conditions for trypsin immobilization in dioxane were calculated as: 0.12 $g_{CDI}/g_{particle}$, 82 min activation time and 1 h coupling time.

Table 2.2 Response factor models as function of activator concentration, activation time and coupling time.

Response factor	Model type	Model significa-nce	significant model terms	Model equation	R^2	Model -Data CV / %
protein coupling yield	2FI	significant	B, C, BC	= 114.81 * -0.49 * B -4.94 * C 0.10 * B * C	0.60	8.35
activity coupling yield	linear	significant	C	= 23.57 * 0.02 * A 0.01 * B -2.62 * C	0.74	15.23
rel. specific activity	2FI	significant	B, C, BC	= 13.17 * 0.26 * B 0.40 * C -0.06 * B * C	0.68	27.94
reusability	2FI	significant	C, AC	= 106.39 * -0.47 * A -5.16 * C 0.12 * A * C	0.30	5.64

A: activator conc., B: activation time, C: coupling time. 2FI: two-factor-interaction.

A verification of model-predicted response values by experimental values at optimum conditions was conducted. Table 2.3 shows the results of this comparison. For better comparability a mean value of protein input within statistical design values and actual achieved protein input in verification experiment are given. The resulting deviation of experimental and model-predicted values may be based on different protein loadings. This factor may act as a strong disturbance variable not taken into account in the present response factor models. Actual protein loading may have a strong influence on activity of particle-bound enzyme, when mass transport effects occur. Nevertheless, considering a correlation variance of the model to the actual design values shown in Table 2.2, a variance lower or equal to this value may be considered as a good prediction. With this prerequisite, models for activity coupling yield, relative specific activity and reusability are applicable to this immobilization process. However, further influence factors for immobilization in dioxane must be evaluated in a one-factor-at-a-time approach.

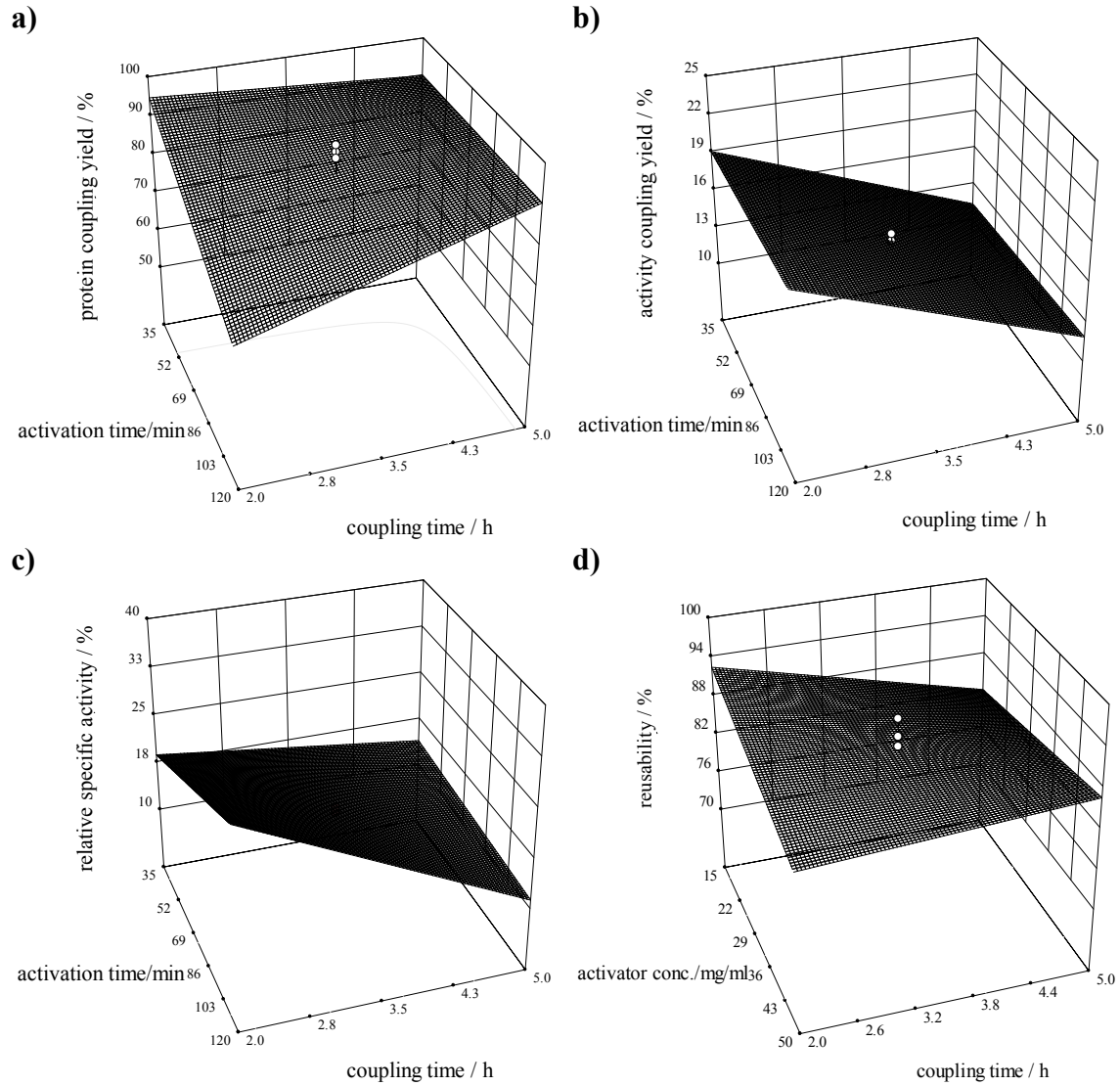


Figure 2.3. Response surface model graphs for influence of activating time, coupling time and activator concentration on protein coupling yield (a), activity coupling yield (b), relative specific activity (c) and reusability (d).

Table 2.3 Comparison of predicted and experimentally obtained results after optimization. Experimental verification was conducted at optimum conditions: A=3 mg/ml, B=82 min, C=1 h.

	protein input $g/g_{particle}$	protein coupling yield/ %	activity coupling yield / %	relative specific activity / %	reusability / %
predicted	0.012	77.9	21.6	30.2	100
experimental	0.021	89.74	18.10	20.23	85.39
CV / %	38.82	9.99	12.49	27.97	11.15

A: activator concentration, B: activation time, C: coupling time.

2.4.4. One-factor-at-a-time immobilization optimization

2.4.4.1. Influence of pH on trypsin lyophilizates immobilization

As enzymes are known to preserve the ionization state of the last aqueous solution in organic solvent, an important factor for immobilization of trypsin in micro-aqueous organic medium is the pH of the aqueous solution from that trypsin is lyophilized. Trypsin was provided as an aqueous solution in 10 mM HCl with an approx. pH 2. In order to investigate the influence of indirect pH variation the trypsin stock solution was mixed in different ratio with a 10 mM NaOH solution, lyophilized and coupled onto magnetic particles in dioxane. The activity of resulting immobilizates was measured in synthesis reaction. The resulting influence of trypsin stock/NaOH-ratio on synthesis activity coupling yield and relative specific activity is depicted in Figure 2.4. The activity of immobilized trypsin decreases with NaOH addition, indicating that the optimum volumetric ratio of trypsin stock and NaOH lies between 0 and 1. Due to solid state of the enzyme, the pH of the resulting lyophilizates could not be determined.

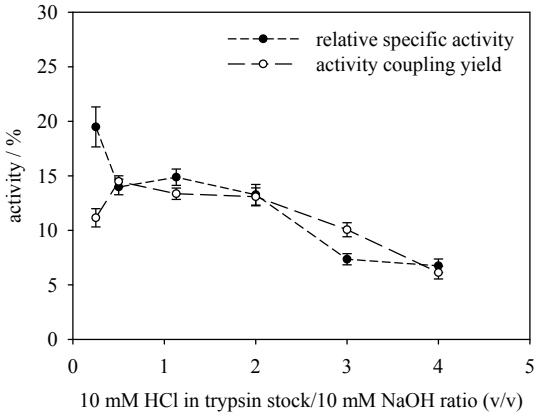


Figure 2.4. Relative specific activity and activity coupling yields of immobilizates in dependence of trypsin stock/NaOH ratio prior to lyophilization.

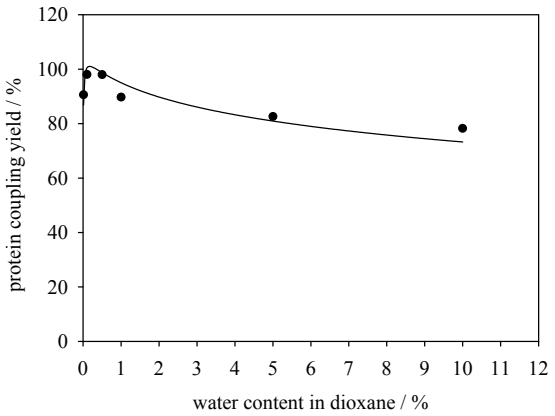


Figure 2.5. Protein coupling yield of trypsin immobilization onto M-PVA in dioxane containing variable water content (given as v/v). Data points were fitted using log normal three parameter equation.

2.4.4.2. Influence of water content in dioxane on protein coupling and carrier activity during immobilization

The water content in dioxane (v/v) during enzyme coupling was elaborated as a significant factor for trypsin immobilization. Trypsin was used in lyophilized form in order to control the water content in dioxane and conserve the enzymatic ionization state from the last aqueous solution during protein

coupling step. Trypsin was used in 0.025 $g_{trypsin}/g_{particle}$ concentration. The trypsin suspension in different dioxane/water solutions exhibited an opaque appearance indicating the enzyme is not entirely soluble in dioxane. After particle introduction and coupling step the enzyme suspension in dioxane appeared clear. Higher water contents lead to faster hydrolysis of CDI-activated carboxylic acids, which has a negative effect on the protein coupling yield (Figure 2.5). Furthermore, the water content influences the solubility and the conformation of the enzyme during the coupling step. Higher water contents may lead to water saturation of enzyme crystals in organic solvent making the enzymes' conformation more similar to a fully aqueous environment (Russell *et al.* 1992). By that the ionization state from lyophilization is altered when organic solvent contains higher amount of water. Furthermore, as the magnetic particles feature a polar surface the presence of enzyme in dioxane with high water content may prevent the adsorption step of the enzyme to the particles surface required for subsequent covalent binding.
Figure 2.6 shows the correlation between carrier activity and water content in dioxane. In the work of Zhu et al. (2011) a water content of 0.4% in dioxane was shown to be optimal for trypsin immobilization onto chitosan microspheres. For immobilization of *C. antarctica* lipase in isooctane an optimum water content of 1.4% (v/v) for residual activity was found by (Sun *et al.* 2010). In this work the data regression shows an optimum protein coupling yield between 0.1 and 0.5% of water in dioxane, while the highest carrier activity was found at 1.2% of water in dioxane. For further experiments a water content of 1% during enzyme coupling step was chosen for best coupling and activity yields.

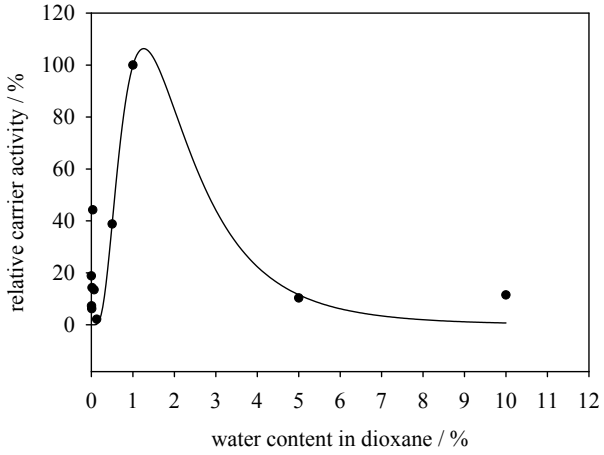


Figure 2.6. Relative carrier activity of trypsin covalently immobilized onto M-PVA in dioxane containing variable water content. Data points were fitted using log normal three parameter equation.

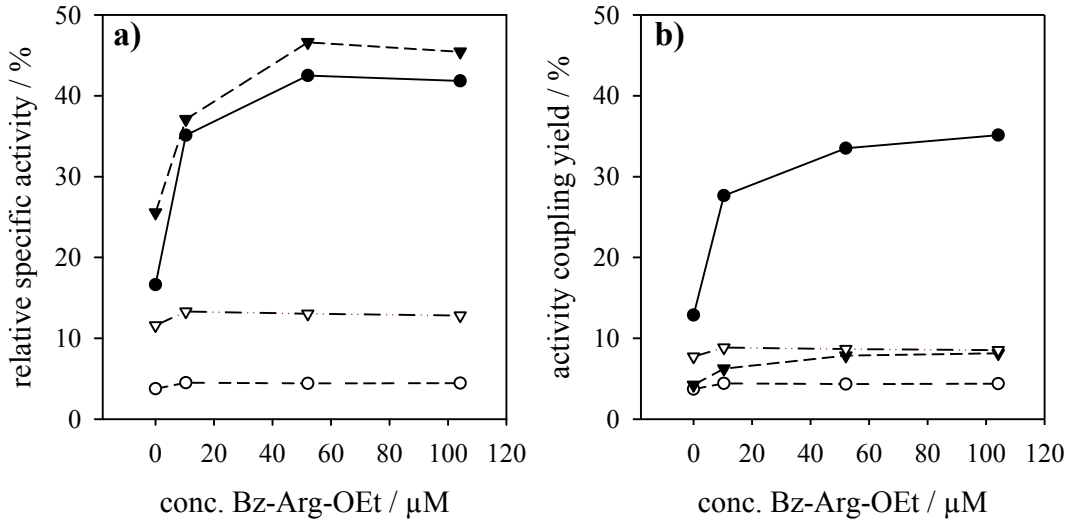


Figure 2.7. Relative specific activity (a) and activity coupling yield (b) in synthesis (▼,▽) and hydrolysis (●,○) reactions of immobilizates in dependency of Bz-Arg-OEt concentration in enzyme coupling medium prior to organic (black) and aqueous (white) immobilization.

2.4.4.3. Substrate imprinting

Previous works report on possible substrate imprinting of enzymes for activity or specificity improvement (Peißker and Fischer 1999; Furukawa *et al.* 2002; Nie *et al.* 2013). However, molecular imprinted trypsin has not been used for immobilization in organic solvent to date. In this work Bz-Arg-OEt was used as an organic and water-soluble trypsin substrate for imprinting prior to enzyme coupling onto magnetic particles in dioxane. Different concentrations of Bz-Arg-OEt in protein coupling medium were used starting with an equimolar trypsin/Bz-Arg-OEt ratio with Bz-Arg-OEt concentration of 10.41 µM. Immobilization was conducted in dioxane and buffer medium as is described in methods with addition of 10 µl Bz-Arg-OEt solution in dioxane or buffer to 1 ml of trypsin in dioxane or buffer. The mixtures were incubated for 2 min and subsequently added to activated magnetic particles. Activity measurements of resulting immobilizates were done in synthesis and hydrolysis reactions. Figure 2.7 a) and b) shows the influence of Bz-Arg-OEt concentration on relative specific activity and activity coupling yield of trypsin immobilized in different media. For aqueous immobilization method a minor improvement of activity by 1-2% with Bz-Arg-OEt introduction may be achieved in synthesis and hydrolysis reactions. This low effect may be explained by Bz-Arg-OEt conversion in aqueous medium. As the enzyme is capable of cleaving the substrate, the substrate and resulting products may

leave the active site without preserving the active structure of trypsin upon immobilization. In micro-aqueous organic medium such as dioxane, however, the substrate may bind to the active site and remain bound, for a lack of water in the surrounding medium prevents the hydrolysis of the substrate. This is shown in the results in Figure 2.7 a) and b). The synthesis activity coupling yield of trypsin immobilized in dioxane increases with Bz-Arg-OEt introduction from 12.86 to 35.84% and the relative specific activity increases from 16.61 to 42.51%. Within hydrolysis reaction type the increase of dioxane immobilization with Bz-Arg-OEt is less prominent with an enhancement of activity coupling yield from 4.22 to 8.14% and relative specific activity from 25.55 to 45.55%. In dioxane immobilization method substrate imprinting has shown to be a promising tool for trypsin activity retention upon immobilization.

2.4.4.4. Influence of different protein loadings on peptide hydrolysis and synthesis of dioxane and aqueous immobilized trypsin

Since the immobilization of trypsin in organic media has been rarely reported, a comprehensive comparison to a more common aqueous covalent immobilization technique on magnetic micro-particles is needed in order to assess the elaborated method in a peptide synthesis and hydrolysis reactions. The protein input for each immobilization method was varied between 0.001 and 0.133 $g_{protein}/g_{particle}$. Immobilization in organic solution was conducted at 1% (v/v) water in dioxane at optimum conditions. For a comprehensive assessment of the two techniques response factors such as protein coupling yield, relative specific activity and activity coupling yield were calculated for peptide synthesis as well as peptide hydrolysis assays and plotted against protein loading in $g_{protein}/g_{particle}$. In Figure 2.8 a) the binding of protein over protein input is shown. Within aqueous immobilization method the binding increases almost linearly with protein input. By dioxane immobilization a similar progress of protein binding with protein input up to a value of 0.02 $g_{protein}/g_{particle}$ is shown. With protein inputs above this value the protein binding reveals saturation where the amount of bound protein does not exceed 0.02 $g_{protein}/g_{particle}$. Figure 2.8 b) shows the results of protein coupling yield for both tested methods. Within aqueous method protein loadings of up to 0.04 $g_{protein}/g_{particle}$ with 95% and higher protein coupling yields are achievable and

with protein inputs higher than 0.04 $g_{protein}/g_{particle}$ the protein coupling yield diminishes to 80%. For the dioxane immobilization method the protein coupling yield values are significantly lower. Highest protein coupling yields of 80-84% are reached at 0.01-0.02 $g_{protein}/g_{particle}$. The amount of bound trypsin molecules in dioxane is limited by the distribution and solubility of trypsin in this solvent. This result is confirmed by the work of Malmsten and Larsson (2000). Trypsin was immobilized in a water-in-oil microemulsion. The stability of trypsin in this emulsion was limited by the maximum protein concentration that could be applied in the stated method (Malmsten and Larsson 2000).

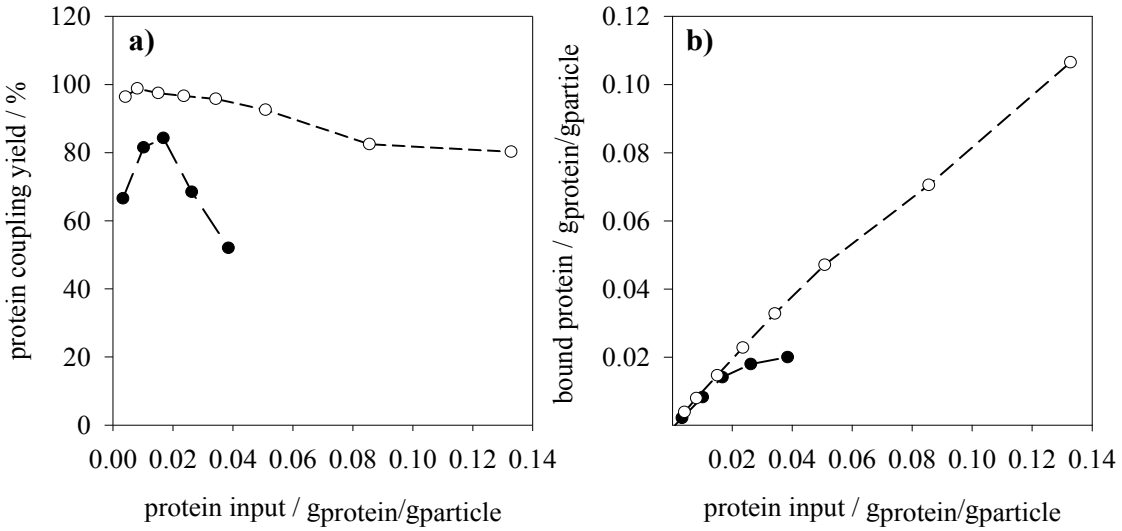


Figure 2.8. (a) Bound protein in $g_{protein}/g_{particle}$ and (b) protein coupling yield as function of protein input for immobilization of trypsin onto M-PVA in dioxane (●) and MES buffer (○).

The synthesis and hydrolysis activity coupling yields for dioxane and aqueous immobilization methods are depicted in Figure 2.9 and Figure 2.10. Within synthesis reaction (Figure 2.9) the activity yields of different protein loadings by aqueous method remain approximately constant at values of 6-8%. However, the dioxane method may reach activity coupling yields of up to 20% showing higher values than the aqueous method within a protein input range of 0.004-0.04 $g_{protein}/g_{particle}$. The activity coupling yield for the dioxane method experiences a drop with protein input of >0.012 $g_{protein}/g_{particle}$. One reason for this behavior is the decreasing protein loading yield in dioxane immobilization which leads to saturation in protein loadings above 0.02 $g_{protein}/g_{particle}$ protein input (Figure 2.8 a). This immobilization method presents a similar behavior in the hydrolysis reaction to the synthesis reaction (Figure 2.10). The maximum activity coupling yield amounts to 20%. For the aqueous immobilization method a decrease in activity coupling yield with increasing protein input from

57 to 5.6% in hydrolysis was observed. Since the protein coupling yield in aqueous method amounts to 95-100% up to a protein input of 0.04 $g_{trypsin}/g_{particle}$ the decrease in activity coupling indicates a limitation of substrate transport in the medium. Especially, as the introduced substrate concentration of 1 mM BAPNA in this reaction is below the stated K_m value of trypsin for BAPNA of 1.5 mM (Johnson *et al.* 2002).

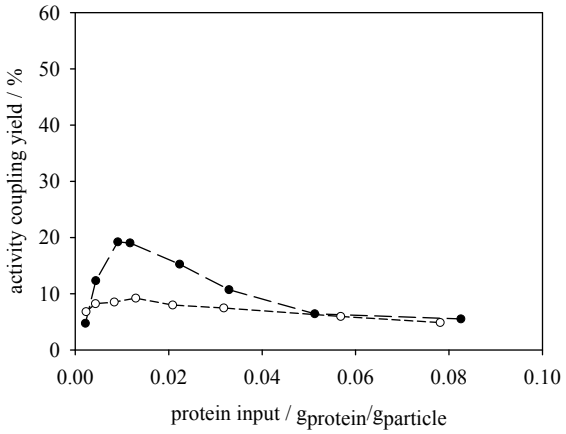


Figure 2.9. Effect of protein input on peptide synthesis activity coupling yield of trypsin immobilized in dioxane (●) and MES buffer (○).

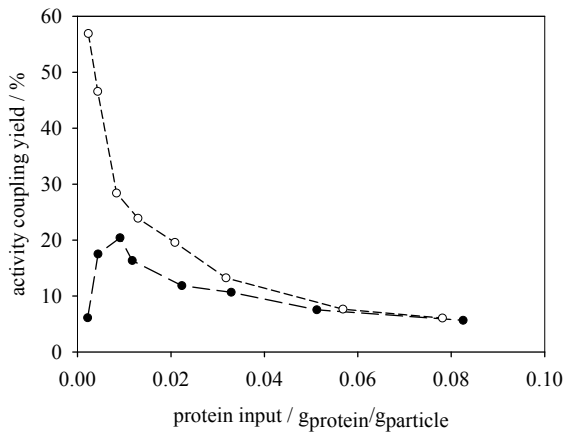


Figure 2.10. Effect of protein input on peptide hydrolysis activity coupling yield of trypsin immobilized in dioxane (●) and MES buffer (○).

Considering the relative specific activity as a function of actual bound protein in $g_{trypsin}/g_{particle}$ the two methods show a diverging behavior in the peptide synthesis reaction. The synthetic activity is depicted dependent on bound protein in Figure 2.11. The results of aqueous immobilization indicate a rapid decrease of specific activity within very low protein concentrations of <0.004 $g_{protein}/g_{particle}$ reaching a constant value of 4-5% above this protein loading. Within the dioxane immobilization method the relative specific activity starts with 100% at 0.002 $g_{protein}/g_{particle}$ decreasing to 20% at 0.02 $g_{protein}/g_{particle}$. Considering the specific hydrolysis activities the two methods show stronger convergence (Figure 2.12). In the dioxane immobilization method a specific activity drop from 88% at 0.002 $g_{protein}/g_{particle}$ to 23.3% at 0.02 $g_{protein}/g_{particle}$ occurs, while in the aqueous method the specific activity starts at 33.3% for 0.004 $g_{protein}/g_{particle}$ and decreases to a value of 4.4% at 0.11 $g_{protein}/g_{particle}$. As mentioned above, the main reason for the decrease in specific immobilized trypsin activity within synthesis and hydrolysis reactions may be the limitation of substrate available to the bound enzyme on the particle surface. Within the synthesis reaction the immobilizate is less

susceptible to mass transport limitation, as the catalysis reaction is slower than in hydrolysis and higher substrate concentrations (10 mM Bz-Arg-OEt, 1.5 U/$mg_{free\ trypsin}$) are used. Compared to the synthesis reaction, the reaction rate in hydrolysis is higher and the concentration of substrate is lower (1 mM BAPNA, 2.7 U/$mg_{free\ trypsin}$). Furthermore, it is conceivable, that the synthesis reaction is more substrate specific and dependent of a certain enzyme conformation state. The BAPNA hydrolysis reaction may occur at slow, basic levels, even without introduction of a biocatalyst and may hence require a less conserved enzyme conformation. Since organic immobilization may preserve a more active conformation of trypsin compared to the aqueous method, this effect may be more prominent in the synthesis reaction. Consequently, the results show that the use of organic method for immobilization of trypsin at protein loading of 0.01 $g_{protein}/g_{particle}$ and lower may improve the conversion of substrates in synthesis and hydrolysis reactions, compared to an aqueous covalent binding method. These findings are supported by observations made by Stark and Holmberg (1989) who immobilized lipase from *Rhizopus sp*. in aqueous and organic systems in order to perform hydrolysis and transesterification reactions. The authors stated that lipase immobilized under organic conditions was more active in transesterification reaction whereas lipase immobilized in buffer showed no activity at all. In hydrolysis reaction the differences between the compared methods were less pronounced (Stark and Holmberg 1989).

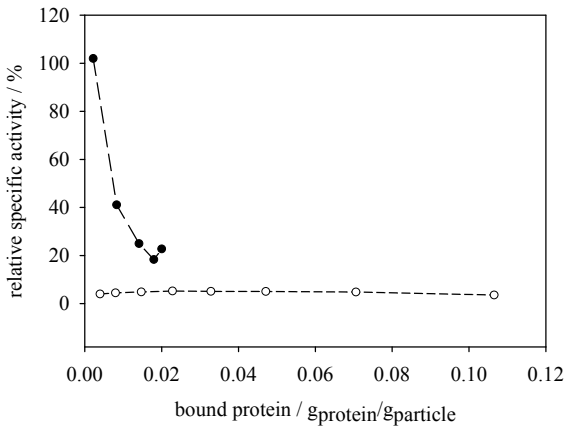


Figure 2.11. Effect of protein input on relative specific peptide synthesis activity of trypsin immobilized in dioxane (●) and MES buffer (○).

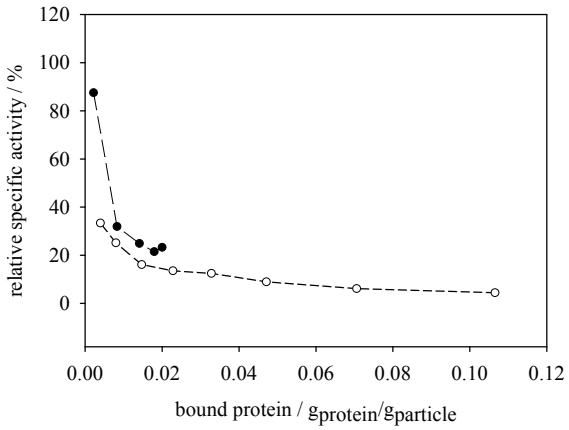


Figure 2.12. Effect of protein input on relative specific peptide hydrolysis activity of trypsin immobilized in dioxane (●) and MES buffer (○).

2.4.4.5. Reusability of trypsin immobilized in dioxane

In order to determine the stability of organic medium immobilized trypsin in repeated peptide synthesis and hydrolysis reactions, immobilizates produced at optimum conditions in dioxane with a 0.02 $g_{trypsin}/g_{particle}$ protein loading were used. Peptide synthesis reaction results are shown in Figure 2.13. After ten reuse cycles the relative carrier activity in peptide synthesis reaction remains at 90% of initial activity. The hydrolysis activity remains at 87% after ten cycles. These results show higher operational stability compared to earlier works. Covalent immobilized trypsin in organic solvent retained 68% of its hydrolytic activity after seven cycles (Zhu *et al.* 2011). Porcine pancreatic lipase immobilized covalently in organic media retained 63% of initial activity after five runs in butyl octanoate synthesis (Ozturk and Kilinc 2010). Earlier works reported on the reusability of enzymes immobilized in organic media by adsorption. De Castro *et al.* (1999) stated a remaining activity of 40% after three repeated batches of butanol conversion by porcine pancreatic lipase (de Castro *et al.* 1999). In the work of Sun *et al.* (2010) a synthetic activity of 57% remained after seven repeated conversions with immobilized lipase from *C. antarctica,* but only 25% of hydrolytic activity remained after seven cycles (Sun *et al.* 2010). Possible reasons for activity decrease during repeated application of immobilized enzymes are enzyme desorption from the carrier or activity loss due to enzyme inactivation in reaction media. Apparently, covalent binding of enzymes in organic medium circumvents possible desorption of enzyme from the carrier in other organic or aqueous media. In addition, the activity of the immobilized enzyme remains more stable compared to the initial value.

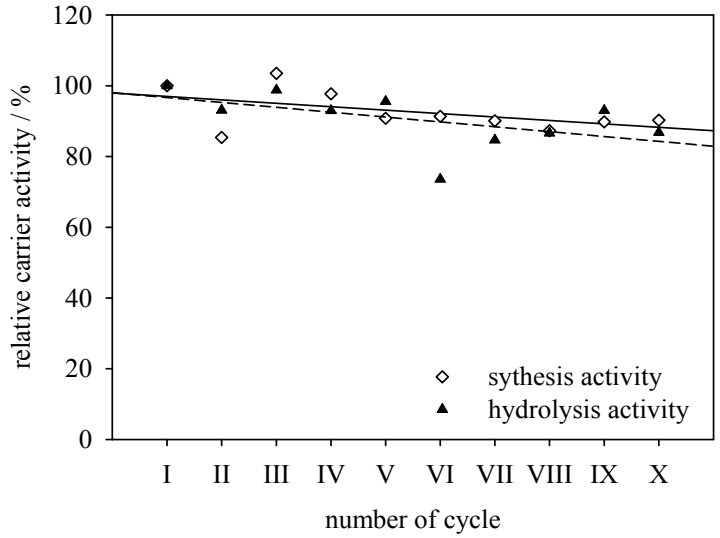


Figure 2.13. Reusability of trypsin immobilized onto M-PVA in organic solvent in synthesis reaction (◇) and hydrolysis reaction (▲). One cycle of synthesis reaction corresponds to one hour of peptide synthesis reaction in 80% ethanol at 20°C, one cycle of hydrolysis reaction corresponds to 30 min in Tris buffered solution pH 8 at 30°C. Linear trend lines show possible end values of activity for synthesis (—) and hydrolysis (– – –) reactions.

2.5. Summary Chapter 2

In this chapter a method for covalent trypsin immobilization on magnetic particles in organic solvent was developed. The method development included immobilization solvent screening, statistical experiment design and one-factor-at-a-time optimization. The optimization was directed onto improvement of peptide synthesis activity of immobilized trypsin. The screening revealed dioxane as most suitable immobilization medium. Within this immobilization method important influence factors such as particle activation and enzyme coupling time, water content in dioxane, indirect pH of trypsin lyophilizate, protein loading and substrate imprinting were identified. The compiled method was compared to a commonly used immobilization of trypsin in buffered aqueous solution on same particle sort. The results indicate an up to sixfold improved specific synthesis activity of immobilized trypsin in dioxane immobilization compared to aqueous medium. In hydrolysis reaction these two methods show similar relative specific activities. One of the most important findings in this section is the difference in hydrolysis and synthesis activities of trypsin depending on immobilization medium. Trypsin immobilized within both methods shows a good reusability with an activity loss of approx. 10% after 10 cycles in both reaction types.

3. Evaluation of chemical nature of carrier and binding

This chapter is partially based on the manuscript

EFFECT OF SPACER MODIFICATION ON ENZYMATIC SYNTHETIC AND HYDROLYTIC ACTIVITIES OF IMMOBILIZED TRYPSIN

Julia Andre[a], David Saleh[a], Christoph Syldatk[a], Rudolf Hausmann[b]

[a]Institute of Process Engineering in Life Science, Section II: Technical Biology, Karlsruhe Institute of Technology, Karlsruhe, Germany [b]Department of Bioprocess Engineering, Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

---

The final publication is available at http://www.elsevier.com/locate/molcatb

Bibliographic details:

Article in press in *Molecular Catalysis B: Enzymatic* January 2016, Elsevier

DOI: 10.1016/j.molcatb.2016.01.003

Authors' contributions to the publication:

Julia Andre planned the experiments, conducted the experimental work, interpreted the results, and wrote the manuscript.

David Saleh carried out the experiments for comparison of diaminoalkyl and aminoalkyl acid spacers.

Christoph Syldatk supervised the project, gave theoretical and practical input for experiments, and contributed to scientific discussions.

Rudolf Hausmann supervised the project, proof-read the manuscript, and provided scientific input for this study.

3.1. Binding enzymes via spacers

Covalent binding of enzymes onto carriers is a suitable method for enzyme stabilization and reutilization in bioreactors (Goddard and Hotchkiss 2007; Sheldon 2007). The binding nature prevents enzymatic leakage into bulk medium and thus expensive inactivation and separation methods. However, the immobilization process leads to lower activity and changed specificity and operational optimum conditions compared to the free enzyme (Cao 2005). Possible reasons for these shortcomings are conformational changes of enzyme on the carrier surface, steric hindrance of the enzymes or mass transfer effects.

The development of immobilization techniques offers several possibilities for adjustment to enzymes' application demands (Garcia-Galan *et al.* 2011; Rodrigues *et al.* 2012). The physical and chemical properties of carrier surface, enzyme or immobilization media and conditions may be customized. The surface properties of the enzyme may be adjusted by spacer chains leading to lower or higher hydrophobicity, distance to enzyme molecule or surface charges thereby altering the interactions with the enzyme molecule.

The application of low and high molecular weight spacers may be categorized by the purpose of enzyme application or investigation. One reason for spacer introduction onto carrier surfaces is the conversion of a high-molecular-weight substrate which is unable to reach the enzymes' active site for example for protein digestion in food or for generating size controlled peptide chains (Itoyama *et al.* 1994; Penzol *et al.* 1998; Yamamoto *et al.* 2005; Hinterwirth *et al.* 2012). Another purpose of spacers is the unfavorable surface polarity of the carrier leading to enzymatic denaturation or partitioning effects on the particle surface (Arica *et al.* 2001; Nouaimi *et al.* 2001; Sharon and Puleo 2008; Du *et al.* 2009; Zhang *et al.* 2011). Additionally, preventing steric hindrance of enzymes or bioactive molecules on carrier surface may be a reason for using spacers, in order to regain enzymatic conformational flexibility (De Maio *et al.* 2003; Zhang *et al.* 2014). However, only a few publications deal with spacers as an operative tool for investigating enzymatic specificity in both, hydrolytic and synthetic reactions (Stark and Holmberg 1989; Ozyilmaz 2009). Furthermore, most publications on spacer-bound enzymes deal with only few kinds of spacers representing an extract of different possibilities for spacer-mediated binding.

In this chapter porcine trypsin was used as model enzyme and immobilized onto magnetic micro-particles via different spacer kinds. The effect of spacer functional groups and length was investigated concerning enzymatic synthetic and hydrolytic activities. The nature of enzyme-particle binding was varied. Furthermore, the influence of protein amount bound covalently via spacers was compared to a direct binding method using no spacer. The reusability of spacer-bound trypsin was compared to the spacer-free immobilization in hydrolytic and synthetic reaction types.

3.2. Materials and Methods

Magnetic poly(vinyl alcohol) micro-particles (M-PVA) C22 and SAV2 with a particle size distribution of 1-3 µm were provided by PerkinElmer Chemagen, Baesweiler, Germany. The used C22 beads feature a carboxyl functionalization with a concentration of 950 µmol COOH/$g_{particle}$. M-PVA SAV2 beads feature streptavidin covalently bound on particle surface. 1,4-Dioxane, methanol, ethanol, acetonitrile, 1,6-diaminohexane, 2-(N-morpholino)ethanesulfonic acid (MES), Carbonyldiimidazol (CDI), N,N'-Disuccinimidyl carbonate (DSC), calcium chloride, tris(hydroxymethyl)aminomethane (Tris), bovine serum albumin (BSA) and trifluoroacetic acid (TFA) were purchased from Carl Roth, Karlsruhe, Germany. 1,4-Diaminobutane, 1,8-diaminooctane, 1,10-diaminodecane, 1,12-diaminododecane, 4-aminobutanoic acid, 6-aminohexanoic acid, 8-aminooctanoic acid, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), Nα-benzoyl-L-arginine ethyl ester hydrochloride (Bz-Arg-OEt), Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA), p-nitroaniline (pNA), L-Arginine amide dihydrochloride (Arg-NH_2) and HABA (4-hydroxyazobenzene-2-carboxylic acid)/Avidin reagent were purchased from Sigma-Aldrich, Deisenhofen, Germany. Glutardialdehyde (GDA) was purchased as 50% solution in water from Merck, Darmstadt, Germany. 12-Aminododecanoic acid was purchased from TCI, Eschborn, Germany. EZ-Link NHS-Biotin, EZ-Link NHS-LC-Biotin, EZ-Link NHS-PEG_4-Biotin EZ-Link NHS-PEG_{12}-Biotin were obtained from Thermo Scientific, Rockford, USA. Recombinant porcine trypsin (EC 3.4.21.4) was purchased from Roche Diagnostics, Mannheim, Germany. Nα-Benzoyl-arginyl-arginine amide trifluoroacetate (Bz-Arg-Arg-NH_2) was synthesized by Bachem, Weil am Rhein, Germany. Nα-Benzoyl-L-arginine (Bz-Arg) was obtained from ABCR GmbH, Karlsruhe, Germany. BC assay was performed by a BC (bicinchoninic

acid) Assay Protein Quantitation Kit from Interchim, Uptima, Montluçon, France. HPLC analysis was performed with a standard HPLC device from Agilent 1200 Series, Agilent, Waldbronn, Germany equipped with a reversed phase column (C18 Luna, 5 µm, 250x4.60 mm, Phenomenex, California, USA). Statistical analysis of the results was conducted using GraphPad QuickCalcs Software from GraphPad Software, Inc., La Jolla, USA.

3.2.1. Modification of M-PVA C22 particles

For modification of M-PVA particles via different spacers the particle suspensions were initially adjusted to pH 5.3 and subsequently activated by a 2 $g_{EDC}/g_{particle}$ EDC solution in 100 mM MES pH 5.3 for 6 min at 11 °C. After activation, particles were washed once with MES buffer and twice with methanol for preparation of diaminoalkane coupling and three times with MES buffer for preparation of aminoalkanoic acids coupling. Diaminoalkane spacers were added as a 40 mmol of diaminoalkane/$g_{particle}$ solution in methanol. Aminoalkanoic acids and BSA were solved in 100 mM MES buffer pH 7.1 as a 40 mmol of aminoalkanoic acid/$g_{particle}$ or 11 $mg_{BSA}/g_{particle}$ with the exception of 12-aminododecanoic acid which was poorly soluble in many solvents and was thus solved in 7 M acetic acid solution in water. Spacer solutions and magnetic particles were incubated for 2 h at 18 °C. Diaminoalkane modified particles were washed three times with methanol and three times with 100 mM MES buffer pH 7.1. Aminoalkanoic acid and BSA-modified particles were washed with 100 mM MES buffer pH 5.3 with the exception of 12-aminododecanoic acid which was first washed three times with 7 M acetic acid and subsequently washed three times with 100 mM MES buffer pH 5.3. BSA wash supernatants were used for BSA loading quantification via BC assay.

3.2.2. Covalent immobilization of trypsin onto modified and non-modified magnetic particles

Diaminoalkane spacer-modified particles were activated by a 40 $ml_{25\%\ GDA}/g_{particle}$ solution in water for 3 h at 25 °C. Aminoalkanoic acid and BSA-modified particles as well as non-modified M-PVA C22 particles were activated by a 2 $g_{EDC}/g_{particle}$ solution in 100 mM MES pH 5.3 for 6 min at 11 °C. After activation, all particles were washed twice with 100 mM MES pH 7.1. Trypsin was added to particles as a 25 mg/$g_{particle}$ solution in 100 mM

MES pH 7.1, or at given varying concentrations, and incubated for 30 min at 25 °C. Subsequently, particles were washed with 40 mM Tris buffer pH 9 for three times and stored in 20 mM $CaCl_2$ solution at 4 °C. Washing steps with Tris buffer were applied in order to desorb non-covalently bound trypsin and inactivate remaining EDC-activated carboxyl groups after protein binding. The supernatants as well as all washing solutions after trypsin coupling were used for determination of bound protein via BC assay. Particle concentrations were determined gravimetrically by drying 250 µl of particle suspension after complete immobilization process at 70 °C for at least 16 h.
For immobilization in organic medium the protocol given in Chapter 2, Section 2.3.1 was used.

3.2.3. Biotin binding onto trypsin

EZ-Link-NHS-Biotin and EZ-Link-NHS-LC-Biotin were used as 10 mM stock solutions and EZ-Link-NHS-$PEG_{4/12}$-Biotin as 200 mM stock solutions, both in water free DMF. Biotin stock solutions were used in a 20-fold molar excess to a 2 mg/ml trypsin solution in phosphate buffered saline (PBS) pH 7.2. For biotin coupling the mixture was incubated for 2 h on ice. Subsequently, trypsin was separated by membrane ultrafiltration with a 3 kDa cut-off. Purified trypsin was resuspended as a 2 mg/ml solution in PBS. The biotinylation grade of trypsin was quantified by HABA/Avidin assay, Section 3.2.6.

3.2.4. Affinity binding of biotinylated trypsin onto M-PVA SAV2 particles

M-PVA SAV2 particles, which have streptavidin covalently immobilized onto the surface, were washed for three times with PBS. Biotinylated trypsin was added as a 20 $mg/g_{particle}$ solution and incubated for 30 min at room temperature. After binding, particles were washed for three times with PBS and stored at 4 °C. The amount of bound protein was determined by performing BC assay of supernatants.

3.2.5. Synthetic and hydrolytic activity measurement of free and immobilized trypsin

The synthetic and hydrolytic activity measurements were conducted as given in Chapter 2 in Section 2.3.2.

3.2.6. Quantification of biotin concentration in samples

Quantification of biotinylated samples was performed by using a HABA/Avidin reagent from Sigma according to manufacturer's instructions. In brief, 900 µl of reconstituted reagent were added into a 1 ml cuvette and absorption at 500 nm was read. The reagent was mixed with 100 µl of sample and absorption measurement was repeated. The concentration of biotin in µmol/ml was calculated from differential absorption with an extinction coefficient of 34 $mM^{-1}cm^{-1}$ and 10 as sample dilution factor.

3.2.7. Protein quantification assay

Protein quantification of protein stock solution and supernatants after immobilization were conducted as given in Chapter 2 in Section 2.3.3.

3.2.8. High pressure liquid chromatography (HPLC) analysis of peptide synthesis samples

The method for analysis of dipeptide synthesis samples is given in Chapter 2, Section 2.3.4.

3.2.9. Calculation of of enzymatic activity parameters

Enzymatic activity response parameters (equations (3-1)-(3-3)) could be calculated after product quantification in each individual activity measurement for various immobilization conditions.

$$\text{immobilizate activity} = \left[\frac{U}{g_{particle}}\right] = \left[\frac{\text{units of enzyme activity}}{g_{particle}}\right] \tag{3-1}$$

$$\text{immobilized/free enzyme's specific activity} = \left[\frac{U}{g_{protein}}\right] \tag{3-2}$$

$$\text{relative specific activity [\%]} = 100\% \times \frac{\text{immobilized specific activity } [U/g_{bound\ protein}]}{\text{specific activity free enzyme } [U/g_{protein}]} \tag{3-3}$$

3.2.10. Reusability study

Reusability study was carried out by performing synthetic/hydrolytic activity assay of immobilizates as given in Section 3.2.5. Ten sequential activity

measurement cycles were performed in triplicate. In particular, after a preceding cycle was terminated, the immobilizate was separated by magnetization from the reaction solution and the solution was discarded. Immediately after particle separation step, a new cycle was initiated by introducing a fresh substrate solution to the immobilizate. The reusability was calculated as given in equation (3-4).

$$\text{reusability [\%]} = 100\% \times \frac{\text{immobilized specific activity cycle\#10 } [\text{U}/\text{g}_{\text{particle}}]}{\text{immobilized specific activity cycle\#1 } [\text{U}/\text{g}_{\text{particle}}]} \qquad (3\text{-}4)$$

3.2.11. Statistical analysis

Results presented in this work, were analyzed by GraphPad statistical software performing a conventional t-test. The resulting probability values (p-values) provided information about the significance of the observed differences. p-values were sorted into following significance levels: $p > 0.05$ not significant, $p \leq 0.05$ indicates significant results with one asterisk in figures, $p \leq 0.01$ indicates very significant results with two asterisks, $p \leq 0.001$ indicates highly significant results with three asterisks.

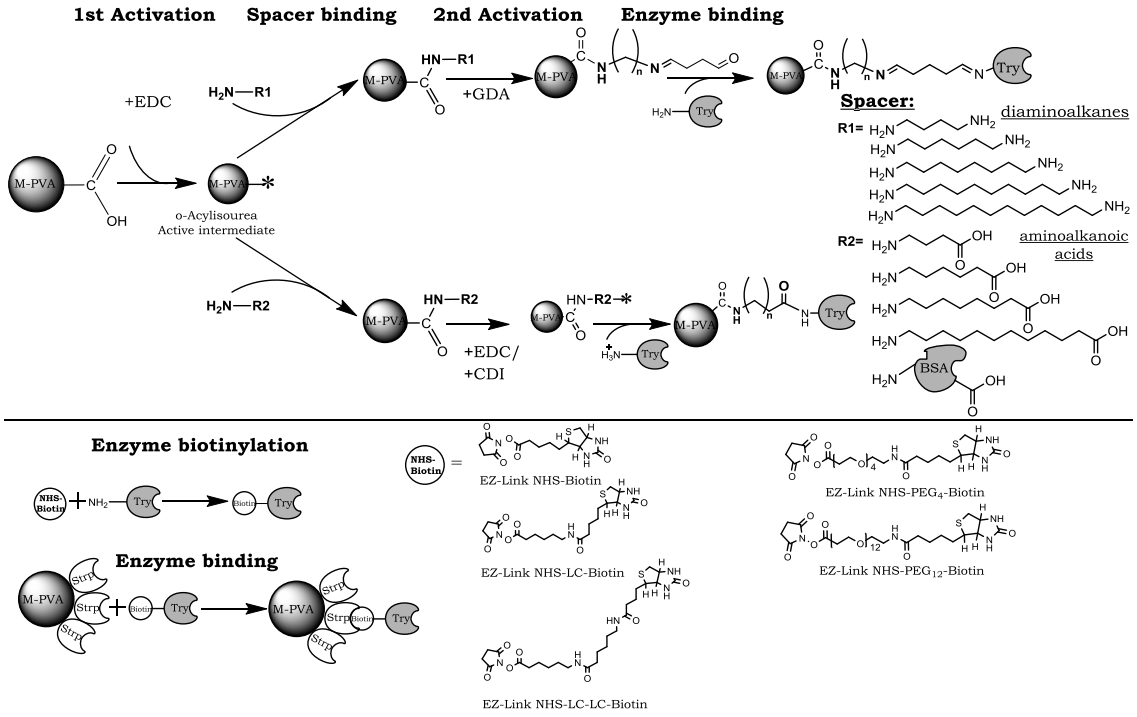


Figure 3.1. Immobilization procedures used in this work: Covalent coupling of trypsin via diaminoalkanes, aminoalkanoic acids and BSA; affinity coupling of trypsin via streptavidin-biotin binding with different spacer inserts.

3.3. Results and Discussion

In this study, a major comparison of different spacers was done investigating their influence on the synthesis and hydrolysis activities of trypsin. Subject of this investigation was the covalent and non-covalent coupling of trypsin to M-PVA C22 and M-PVA SAV2 particles via different spacers differing in their length, hydrophobicity and functional groups. Figure 3.1 depicts schematically covalent and non-covalent spacer coupling and subsequent trypsin binding onto magnetic micro-particles. Table 3.1 summarizes the characteristics of spacers used in this study.

Table 3.1. Characteristics of spacers used in present work for immobilization of trypsin.

Name	Chemical structure	MW / g/mol	Theoretical spacer length / nm	Nature of binding
	no spacer		0.00	covalent
1,4-Diaminobutane	H_2N-$(CH_2)_4$-NH_2	88.15	1.60*	covalent
1,6-Diaminohexane	H_2N-$(CH_2)_6$-NH_2	116.21	1.90*	covalent
1,8-Diaminooctane	H_2N-$(CH_2)_8$-NH_2	144.26	2.20*	covalent
1,10-Diaminodecane	H_2N-$(CH_2)_{10}$-NH_2	172.31	2.50*	covalent
1,12-Diaminododecane	H_2N-$(CH_2)_{12}$-NH_2	200.43	2.80*	covalent
4-Aminobutanoic acid	H_2N-$(CH_2)_3$-COOH	103.12	0.75*	covalent
6-Aminohexanoic acid	H_2N-$(CH_2)_5$-COOH	131.17	1.05*	covalent
8-Aminooctanoic acid	H_2N-$(CH_2)_7$-COOH	159.23	1.35*	covalent
12-Aminododecanoic acid	H_2N-$(CH_2)_{11}$-COOH	215.33	1.95*	covalent
BSA		66463	6.96**	covalent
Biotin		341.38	0.75***	affinity
Biotin-LC		454.54	1.79***	affinity
Biotin-LC-LC		567.7	2.84***	affinity
Biotin-PEG_4		588.67	2.52***	affinity
Biotin-PEG_{12}		941.09	4.86***	affinity

* Respective spacer length was calculated from binding length between spacer atoms.
** BSA length from (Axelsson 1978).
*** Spacer length of biotin conjugates was calculated from chains inserted between NHS and biotin molecule. Spacer length of biotin-molecule was not considered.

3.3.1. Screening for covalent spacer binding method for diaminoalkane spacers

For subsequent comparison of different spacer types containing terminal amino and carboxyl groups, methods for covalent spacer coupling, as well as

trypsin coupling onto spacer had to be evaluated. As method for aminoalkanoic acid spacer binding the same method as for direct covalent trypsin binding was applied and given in Chapter 2, Section 2.3.1. For the best method of binding of diaminoalkane spacers onto M-PVA surface, a combination method screening was performed. Carrier activities of immobilizates in hydrolysis reaction were used as response parameters. The most suitable method for the covalent binding of diaminoalkane spacers was activation with EDC, spacer coupling in methanol and second activation step with GDA followed by trypsin coupling in MES buffer as shown in relative carrier activity values in Table 3.2.

Table 3.2. Method combinations in screening for best suitable covalent coupling method of diaminoalkane spacers, method procedure from left to right.

Activation of carboxy-groups on particle	Spacer coupling medium	Reagent for activation of spacer ends	Trypsin coupling medium	Rel. carrier activity / %
EDC 2 $g/g_{particle}$ in MES buffer pH 5.3	Methanol	GDA*	MES pH 7.1	100
		DSC**		14.0
	MES buffer pH 7.1	GDA		14.7
		DSC		66.2

* solved as 2 $g/g_{particle}$ in acetone.
** as 25% solution in water.
Relative carrier activity refers to a carrier activity value of 300.3 $U/g_{particle}$ in hydrolysis assay.

3.3.2. Influence of different spacers on the synthesis and hydrolysis reactions of trypsin

The degree of trypsin biotinylation measured by HABA/Avidin assay is given in Table 3.3. Up to four biotin molecules may bind to one molecule of streptavidin homo-tetramer. The results show an average biotinylation of 1 to 5 moles of biotin per mole of trypsin. The biotinylation degree is sufficient for every trypsin to bind to at least one streptavidin. Figure 3.2 depicts an overall protein coupling yield at a given protein input for all tested spacer types. Table 3.4 shows the amount of bound protein for each tested spacer shown in Figure 3.2. The highest trypsin binding capacity over a broad protein input range results when no spacer is used. For all tested spacers the resulting protein

coupling yield was lower than for the no spacer condition within the same protein input value. The lowest amount of protein per g of particle compared to the method without spacer could be bound via streptavidin-biotin affinity coupling. From Table 3.4 the protein binding capacity for the tested spacer groups is as follows: biotin spacers < diaminoalkane spacers < BSA < aminoalkanoic acids.

Table 3.3. Measurement results of HABA-assay for determination of biotin molecules covalently bound per mole of trypsin.

Biotin derivative	$mol_{biotin}/mol_{protein}$
Biotin-Trypsin	2.37
Biotin-LC-Trypsin	2.67
Biotin-LC-LC-Trypsin	0.94
Biotin-PEG_4-Trypsin	3.81
Biotin-PEG_{12}-Trypsin	5.35

Table 3.4. Amount of bound protein in spacer-free and spacer-bound immobilization method for further relative specific activity comparison.

Spacer	Bound protein / $g_{protein}/g_{particle}$	Bound protein of corresponding covalent binding method without spacer / $g_{protein}/g_{particle}$
H_2N-$(CH_2)_4$-NH_2	0.0045	0.0088
H_2N-$(CH_2)_6$-NH_2	0.0047	
H_2N-$(CH_2)_8$-NH_2	0.0063	
H_2N-$(CH_2)_{10}$-NH_2	0.0097	
H_2N-$(CH_2)_{12}$-NH_2	0.0084	
H_2N-$(CH_2)_3$-COOH	0.0180	0.0173
H_2N-$(CH_2)_5$-COOH	0.0160	
H_2N-$(CH_2)_7$-COOH	0.0175	
H_2N-$(CH_2)_{11}$-COOH	0.0185	
BSA	0.0139	0.0130
Biotin	0.0006	0.0008
Biotin-LC	0.0010	
Biotin-LC-LC	0.0017	
Biotin-PEG_4	0.0009	
Biotin-PEG_{12}	0.0012	

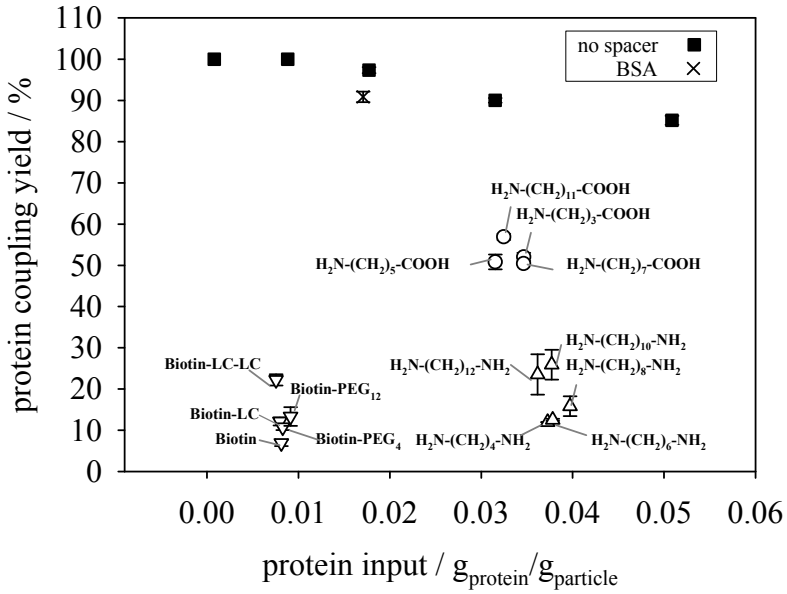


Figure 3.2. Protein coupling yield of different spacer types in comparison to the no spacer condition: initial method without spacer (■), diaminoalkane spacers (△), aminoalkanoic acid spacers (○), BSA (×) and streptavidin-biotin spacers (▽).

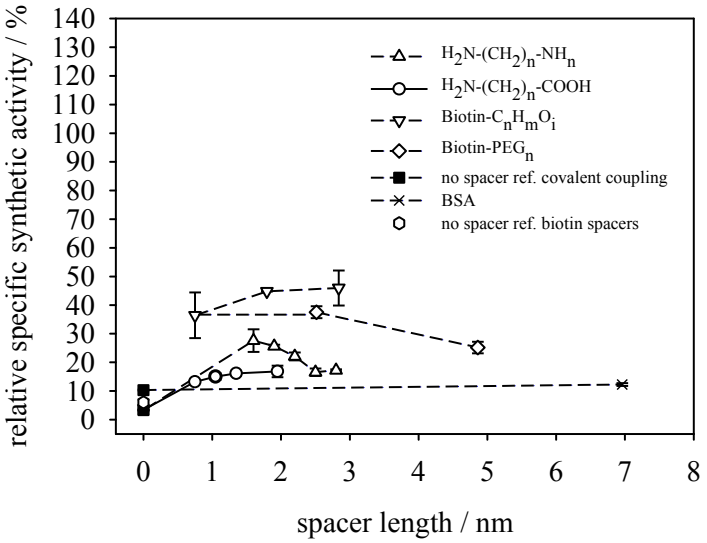


Figure 3.3. Variation of the relative specific synthetic activity of magnetic particle bound trypsin with spacer length. At zero spacer length reference points of the initial covalent coupling method using no spacer within similar protein loading range as in the respective spacer group are depicted (black square, white hexagon).

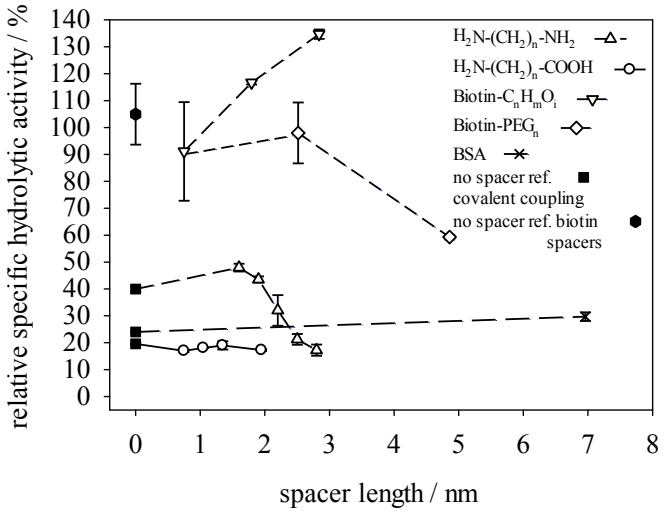


Figure 3.4. Variation of the relative specific hydrolytic activity of magnetic particle bound trypsin with spacer length. At zero spacer length reference points of the initial covalent coupling method using no spacer within similar protein loading range as in the respective spacer group are depicted (black square, black hexagon).

In order to evaluate synthetic and hydrolytic activities of spacer-bound trypsin in comparison to the no spacer condition, activity measurements were conducted. Table 3 refers the bound protein amount of spacer-bound trypsin to a no spacer condition applied in the activity measurement. The variation of the resulting specific activities with spacer length is shown in Figure 3.3 and Figure 3.4. For better comparability, the relative specific activity of spacer-free immobilization is depicted at zero spacer length within similar bound protein amount as the respective spacer group. Means of the no spacer condition were compared to the highest resulting activity mean of a spacer group within the standard deviations depicted in the figures giving a significance level of the results.

Using diaminoalkane spacers resulted in an increase of relative specific synthetic activity compared to that of the spacer-free method of about 24% (Figure 3.3). In the case of hydrolytic activity, the increase amounted to 8% (Figure 3.4). However, with increasing spacer length, the specific synthetic and hydrolytic activities decrease indicating a reversing effect of spacer length on synthetic activity. As diaminoalkane spacers feature amino groups on both ends of the spacer, it appears possible that one spacer is coupled across two activated carboxyl groups during the spacer coupling step. Larger diaminoalkane spacers would tend to crosslink carboxyl groups on and between the particles. This effect may lower the protein coupling yield and result in lowered carrier bound trypsin activity as was observed. Diaminoalkane spacers were tested for immobilization of trypsin, lipase and

glucose oxidase in previous works (Bulmus *et al.* 1997; Yamamoto *et al.* 2005; De Maio *et al.* 2003; Ozyilmaz 2009). Optimum hydrolysis activity of trypsin was achieved with diaminohexane (Yamamoto *et al.* 2005). Lipase performed synthesis reactions with higher specific activities with more hydrophobic spacers such as diaminohexane (Ozyilmaz 2009). Optimum hydrolytic activity of trypsin was achieved with diaminohexane (Yamamoto *et al.* 2005). The hydrolytic activity of trypsin for BAPNA increased linearly with spacer length up to a chain length of C_6. With spacers ranging from 3 to 12 atoms the hydrolytic activity measurement results are in accordance with results obtained in this study. Higher spacer lengths of up to 28 atoms reached an even higher hydrolytic activity when using hydrophilic spacers based on PEG (Hinterwirth *et al.* 2012).

In experiments with aminoalkanoic acids, the protein amount bound onto particle surface via spacers shows a low variation (Table 3.4). With these modified immobilizates, an increase in relative specific synthesis activity with spacer length of 13% in comparison to trypsin immobilized without spacer could be shown (Figure 3.3). A linear increase in specific activity up to a spacer length of 1.35 nm is observed. Longer spacer chains cause saturation in the relative specific activity. However, a significant correlation between the distance of enzyme to particle and synthetic activity was not found using aminoalkanoic acid spacers (p-values comparing values of different aminoalkanoic acid spacers amount to > 0.05). In hydrolysis reaction, the activity of trypsin immobilized via aminoalkanoic acid spacers was significantly lower than when using no spacer ($p = 0.0014$). Thus, an activity improvement compared to the spacer-free immobilization method could not be shown (Figure 3.4).

Due to known studies of protein-stabilizing effect of BSA as a spacer in enzyme immobilization, it was tested as a spacer for trypsin immobilization (Nouaimi *et al.* 2001, Du *et al.* 2009). Covalent immobilization of trypsin via BSA showed a minor improvement of relative specific synthetic activity by almost 2% (Figure 3.3). Similar results were obtained for hydrolytic specific trypsin activity with an activity increase of 5% (Figure 3.4). However, according to the t-test results calculated for BSA with a p-value of 0.0126 for synthesis reaction type and 0.0182 for hydrolysis, the difference obtained in measured activities between the no spacer condition and BSA as spacer is low but significant. However, the achieved activity increase does not correlate with the

assumed spacer length. Although covalent trypsin coupling to BSA-modified particles occurred at neutral pH and not at the activity optimum of trypsin of pH 8-9, a tryptic digestion of BSA by coupled and free trypsin during the coupling step may not be ruled out. In previous works, BSA was used as spacer for immobilization of trypsin with subsequent hydrolytic activity measurement (Nouaimi *et al.* 2001; Cavalcante *et al.* 2006). For trypsin immobilized on polyester via BSA, a threefold improvement of specific hydrolytic activity was shown. Another study reported that by using an exopolysaccharide as carrier, direct immobilization lead to fourfold higher hydrolytic activity results compared to BSA-linked immobilization. The opposite results in these studies may be accounted to particle properties. Since polyester is regarded as hydrophobic carrier, a beneficial effect on enzymatic stability and activity may be expected once a hydrophilic macromolecule, such as BSA, is attached to its surface. For exopolysaccharides and poly(vinyl alcohol) (this work), which feature hydrophilic surfaces, carrier modification by BSA is less beneficial. An activity increase due to greater distance between enzyme and particle using BSA as spacer, could not be confirmed.

Compared to the no spacer condition, using a spacer strongly increased the synthetic activity of biotinylated bound trypsin by 40% for biotin-LC-LC within the biotin-LC group (p-value = 0.0012) (Figure 3.3). In hydrolysis reaction a significant increase of up to 30% is achieved using biotin-LC-LC as spacer (p-value = 0.0392) (Figure 3.4). Considering the biotin-PEG_n-based spacers, the increase of synthetic activity amounts to 31% (Figure 3.3) using biotin-PEG_4 (p-value = 0.0001), but no significant activity increase is achieved when measuring the hydrolytic activity of this group (Figure 3.4). Different activity behavior with increasing spacer length between the biotin-LC and the biotin-PEG_n groups may be attributed to the character of the respective spacer insert. LC-based spacers display a more hydrophobic character, while PEG spacers are described as highly hydrophilic. It may be presumed that the more hydrophobic spacer character leads to a bound enzyme facing away from this spacer into the bulk fluid. The highly hydrophilic character of the PEG spacers may cause a higher interaction with polar enzyme groups with orientation facing towards the carrier decreasing the favorable spacer effect.

Beside the chemical and physical spacer characteristics of each spacer group, which were considered in this part of the study an important influence factor

on the relative specific activity is the amount of bound protein. Thus, a comprehensive comparison of the protein loading between the spacer groups and the no spacer condition must be conducted.

3.3.3. Protein loading effect on spacer-linked and directly immobilized trypsin

8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane were chosen for further study of the protein loading effect on the synthetic and hydrolytic activities of spacer-bound and spacer-free immobilizates. These spacers were utilized in this part of the study, due to comparable bound protein amounts to the spacer-free method and in order to obtain higher comparability to spacer-free trypsin using covalent attachment to both, the particle and trypsin. The protein input was varied between 0.0008 and 0.19 $g/g_{particle.}$ The resulting bound protein concentrations of the tested spacers and the no spacer condition are shown in Figure 3.5. The bound protein amount of the no spacer condition ranges between 0.0008 and 0.059 $g/g_{particle}$. The maximum achieved protein loadings of the 8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane spacers are 0.038, 0.054 and 0.01 $g/g_{particle}$, respectively. As shown in Figure 3.2, the highest protein loading capacity over a broad protein input range occurs under the no spacer condition. Within all obtained curves in Figure 3.5, a maximum of protein loading value was exhibited. Particles modified with 1,12-diaminododecane show the lowest binding capacity. As was stated above, a possible reason for this low binding is the cross-linking of the functional carboxyl groups by the spacer with lower remaining amount of free amino-groups for covalent enzyme coupling. The amount of bound protein via 8-aminooctanoic acid is lower than for the larger 12-aminododecanoic acid spacer. Possible reason for this may be an incomplete binding of the 12-aminododecanoic acid spacer to the functional groups of the particle. Due to a low solubility of the 12-aminododecanoic acid spacer, the binding is conducted in acetic acid containing medium which may lead to incomplete spacer coupling to the particle surface for its low pH. As a result, only a part of the enzyme is may be bound via spacer, whereas another part is bound directly to the particle.

The influence of protein loading on the relative synthetic and hydrolytic specific activities of spacer-bound and spacer-free enzyme is depicted in

Figure 3.6 and Figure 3.7. The relative specific synthetic activity of spacer-free immobilizate reaches the lowest activity values of 3-6% (Figure 3.6). By using 8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane spacers the relative synthetic specific activity may be increased up to 60%, 22% and 35%, respectively. However, an overall decrease of activity is observed with increasing amount of bound enzyme. This fact is attributed to mass transport limitation occurring when the amount of biocatalyst exceeds the substrate available to the enzyme by diffusion transport. Even at lowest protein loading tested, the relative synthetic activity may not reach 100%. The mass transport limitation may additionally occur in combination with structural changes of the enzyme upon immobilization. As a result, these two effects may not be considered separately when measuring the immobilized enzymatic relative activities. By using 8-aminooctanoic acid and 12-aminododecanoic acid spacers, the synthetic specific activity in comparison to the no spacer condition may be increased within 0.008-0.035 $g/g_{particle}$ bound protein range. Binding trypsin via 1,12-diaminododecane leads to an activity increase by binding a protein amount up to 0.0097 $g/g_{particle}$. Within this experiment, it is shown that when using a spacer, the specific synthetic activity of covalently bound trypsin may be increased up to 10-fold compared to direct trypsin binding within the same protein loading.

The relative hydrolytic specific activities of spacer-bound trypsin are compared to directly bound trypsin within different protein loadings (Figure 3.7). In comparison to measured relative synthetic activities, immobilized trypsin exposes higher hydrolysis activity levels for all tested conditions. Using direct binding method, relative specific hydrolytic activities range between 9.5% and 105%. By using 8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane spacers, the relative synthetic specific activity may reach up to 169%, 102% and 37.6%, respectively. 8-aminooctanoic acid as spacer shows the highest increase of hydrolytic activity compared to the direct binding method over a bound protein range of 0.008-0.017 $g/g_{particle}$. 12-aminododecanoic acid shows similar relative hydrolytic activities as the no spacer condition over the entire range of bound protein. Lower relative hydrolytic activities compared to the no spacer condition were obtained by using 1,12-diaminododecane as spacer. Presumably, the chemical character of the diaminoalkane spacer favors the synthesis activity of the bound trypsin over the hydrolytic activity which may be attributed to the more hydrophobic

character of the functional spacer groups of this spacer compared to the aminoalkanoic acid spacers. This fact may have an adverse effect on the hydrolysis reaction.

Summarizing the protein loading effect under spacer usage, the relative specific activities in both, hydrolysis and synthesis reaction types, were most increased by covalent trypsin binding via 8-aminooctanoic acid attachment. Comparing these results to affinity binding of trypsin via biotin, lowering the bound protein amount to 0.008 $g/g_{particle}$ using 8-aminooctanoic acid may result in even higher relative specific activities (67% in synthesis and 168% in hydrolysis) than when using the affinity coupling method via biotin within the same bound protein amount (46% in synthesis and 135% in hydrolysis) (Figure 3.3 to 3.4 and Figure 3.6 to 3.7).

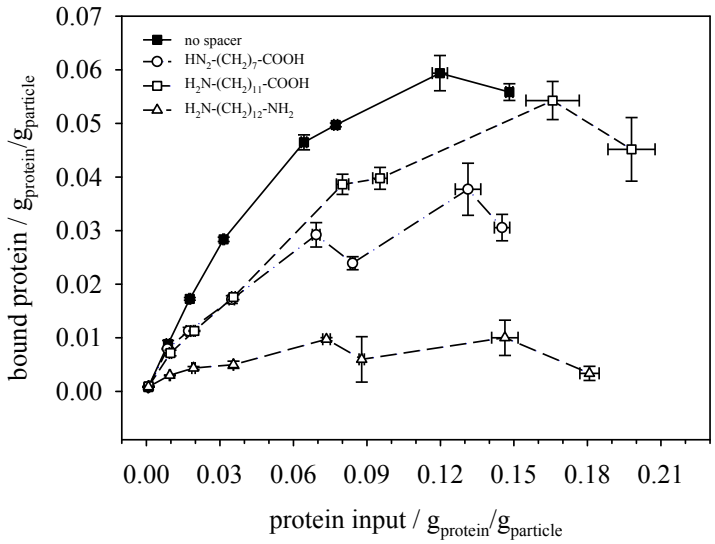


Figure 3.5. Variation of the protein binding capacity with protein input of 8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane-modified and spacer-free magnetic particles-bound trypsin.

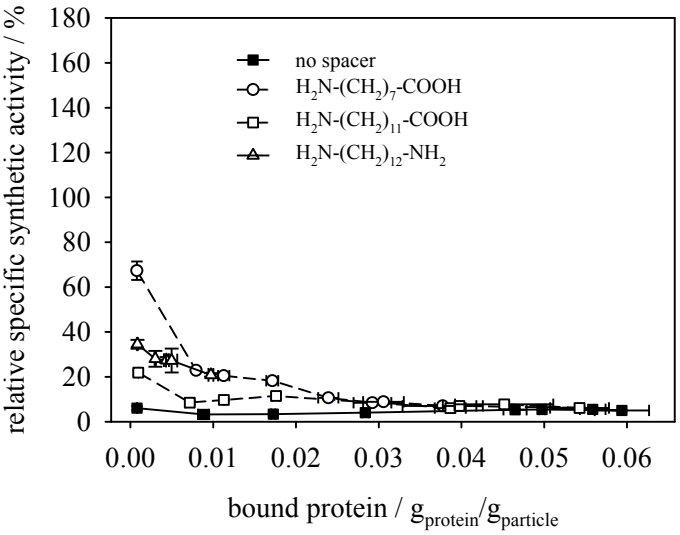


Figure 3.6. Variation of the specific synthetic activity with protein loading of 8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane-modified and spacer-free magnetic particles-bound trypsin.

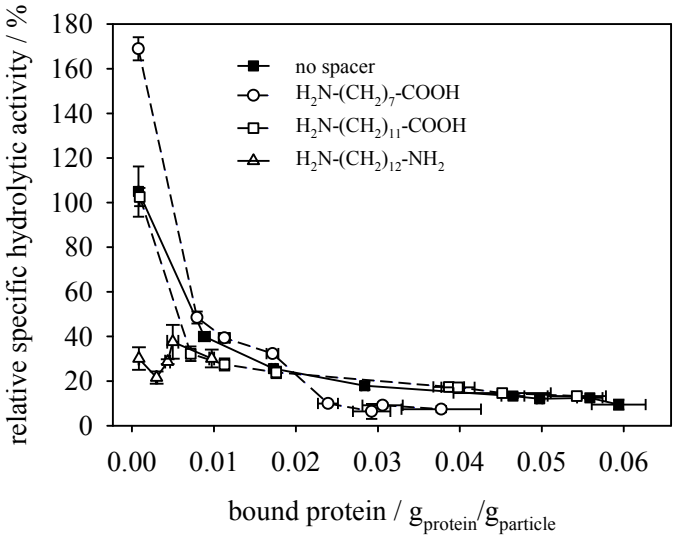


Figure 3.7. Variation of the specific hydrolytic activity with protein loading of 8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane-modified and spacer-free magnetic particles-bound trypsin.

3.3.4. Reusability of trypsin immobilized on different spacers

The experiments aimed to investigate the stability of immobilizates in repetitive use, which is of a particular importance in industrial application of enzyme immobilizates. Lowered reusability of an immobilizate may occur either due to leaching of the enzyme from the particle or deactivation of the enzyme upon application conditions. Repetitive application of 8-aminooctanoic acid, 12-aminododecanoic acid and 1,12-diaminododecane spacer-linked and directly immobilized trypsin were assessed in hydrolytic and synthetic reactions. Figure 3.8 a) depicts the course of absolute specific synthetic activity over ten sequential cycles.

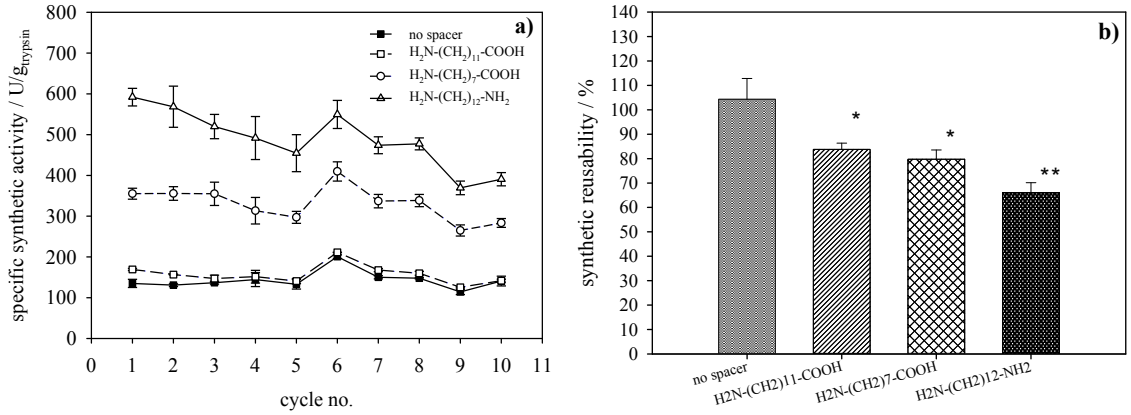


Figure 3.8. a) Course of specific synthetic activity of spacer-modified and spacer-free magnetic particles-bound trypsin over ten cycles. b) Reusability of different spacer magnetic particle bound trypsin in synthesis reaction after ten cycles. Significance of the spacer results compared to the no spacer condition is depicted by using asterisks.

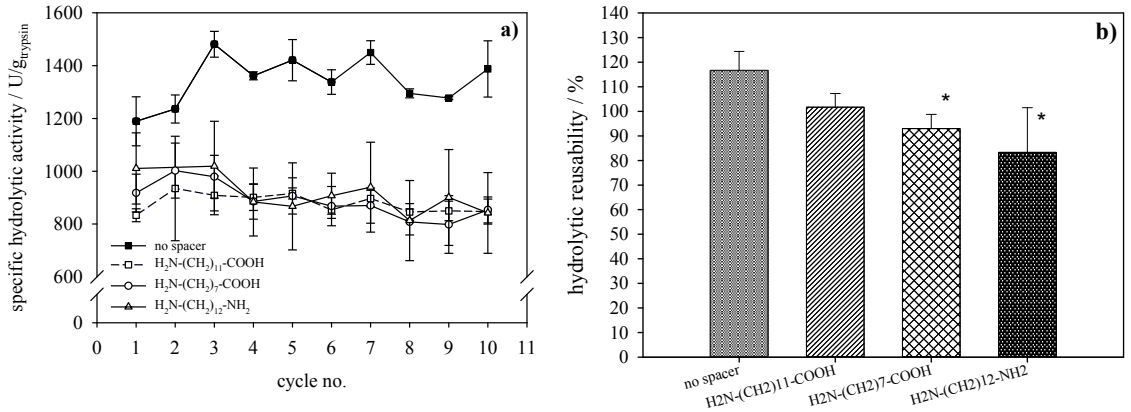


Figure 3.9. a) Course of specific hydrolytic activity of spacer-modified and spacer-free magnetic particles bound trypsin over ten cycles. b) Reusability of different spacer magnetic particle bound trypsin in hydrolysis reaction after ten cycles. Significance of the spacer results compared to the no spacer condition is depicted by using asterisks.

The specific synthetic activity of directly bound trypsin remains at 98% within the first five cycles. Using 12-aminododecanoic acid as spacer, a slight decrease to 83% of in initial activity occurs within the first five cycles. From cycle number one to three, the synthetic trypsin activity immobilized via 8-aminooctanoic acid remains stable and up to cycle five the activity amounts to 84% of initial value. The strongest activity loss occurs within five cycles, when using 1,12-diaminododecane as spacer and amounts to 77% of initial activity. Considering synthesis cycles no. 6 to 10, the specific activity of all tested immobilizates decreases further for all tested spacers. Figure 3.8 b) shows the reusability of the spacer-bound trypsin after ten reuse cycles in comparison to the no spacer condition. The highest synthetic activity stability may be attributed to the no spacer condition followed by 12-aminododecanoic acid, 8-

aminooctanoic acid and 1,12-diaminododecane spacer conditions. Thus, lowest stability of activity within synthesis assay after ten cycles was demonstrated using 1,12-diaminododecane as spacer with a total activity decrease of 34% compared to the initial specific activity value. This fact may be caused by the spacer length or more hydrophobic chemical nature of 1,12-diaminododecane. The loss of activity may occur due to breaks of covalent bonds within the spacer, leaching of adsorptively bound trypsin or higher hydrophobicity of this spacer compared to other tested spacers causing slow enzymatic denaturation.

In order to evaluate the reusability of trypsin immobilizates in hydrolysis reaction type, the immobilizates were applied in ten sequential hydrolysis cycles. Figure 3.9 a) shows the course of the absolute specific hydrolytic activity. Within the no spacer condition no course indicating an activity decrease could be observed. However, all tested spacer-bound immobilizates show a slight decrease of hydrolytic activity within ten cycles. Figure 3.9 b) depicts the hydrolytic reusability of immobilizates related to the initial specific activity value. The highest reusability is demonstrated, when no spacer is used, whereas the lowest reusability value of 83% is shown by 1,12-diaminododecane spacer-attached trypsin.

The evaluation of reusability of immobilizates using different spacers and direct trypsin binding leads to the conclusion that the catalytic stability of trypsin bound via different spacers is slightly lowered by spacer-mediated covalent binding.

3.3.5. Spacer-linked and direct immobilization of trypsin in organic medium

In order to evaluate the effect of spacer application in different immobilization media, and especially to combine the spacer method with organic immobilization method, immobilization via spacer was conducted in dioxane via method from Chapter 2, Section 2.3.1. 12-Aminododecanoic acid was used as spacer. Figure 3.10 and Figure 3.11 show the results of synthetic and hydrolytic specific activities of differently loaded immobilizates with and without spacer introduction. Within synthesis reaction, spacer-bound trypsin shows lower specific activities compared to spacer-free method with differences up to 70% (Figure 3.10). The specific activity values in these two methods show convergence when protein loading increases to 0.018 $g/g_{particle}$ reaching a

difference of only 6%. Within hydrolysis reaction (Figure 3.11), the specific activities of spacer bound and directly immobilized trypsin in organic medium are similar with slightly higher activities at lower protein loading via direct immobilization and identical values within protein loadings > 0.008 $g/g_{particle}$. Hence, the combination of organic immobilization method with immobilization via 12-aminododecanoic acid spacer does not lead to higher specific activities in synthesis and hydrolysis reactions of trypsin. In a previous work lipase immobilized under organic solvent conditions with and without spacer showed no difference in transesterification activity (Stark and Holmberg 1989). However, in hydrolysis reaction type lipase attached via spacer lead to 30% higher residual activity compared to direct immobilization method in organic solvent. Comparing these results to the present work spacer introduction for enzymes immobilized in organic media dealing with hydrophobic substrates such as lipases leads to activity increase, whereas for proteolytic enzymes such as trypsin a combination of organic medium immobilization and hydrophobic spacer did not achieve higher relative specific activities.

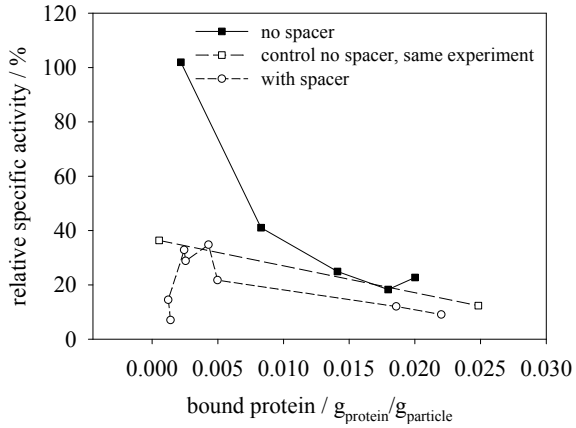


Figure 3.10. Effect of protein loading on specific synthesis activity of 12-aminododecane acid modified (○) and spacer-free magnetic particles in separate measurement (■) and same experiment as modified immobilizates (□). Enzyme coupling step was carried out in dioxane.

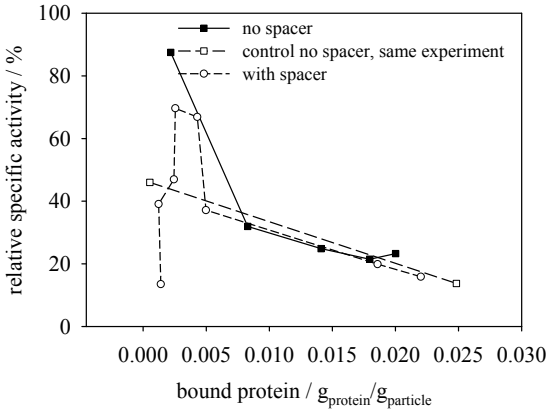


Figure 3.11. Effect of protein loading on specific hydrolysis activity of 12-aminododecane acid modified (○) and spacer-free magnetic particles in separate measurement (■) and same experiment as modified immobilizates (□). Enzyme coupling was carried out in dioxane.

3.4. Summary Chapter 3

In this chapter, a comprehensive comparison of spacer-immobilized and directly bound trypsin was accomplished. The influence of spacer length had a significant impact on the synthesis and hydrolysis activities of trypsin. The specific synthesis activities of trypsin bound via diaminoalkane, aminoalkanoic acid and streptavidin-biotin were higher compared to the initial spacer-free method. Furthermore, within aminoalkanoic acid and streptavidin-biotin spacers, the relative specific synthesis activity increased with spacer length. Varying the protein loading on the spacer-modified particles revealed an improved specific activity in the synthesis reaction over a broad protein loading range. In this chapter a correlation of tryptic synthesis activity and spacer length for different kinds of spacers was shown. A combination of organic immobilization method and spacer-mediated immobilization did not achieve an improvement of trypsin activity in hydrolysis or synthesis assays.

4. Influence of particle size: developing a model peptide oligomerization reaction

In this chapter it is described how immobilization conditions may influence the specificity and product distribution in an oligopeptide synthesis reaction. In particular, the influence of different particle sizes was investigated for peptide oligomerization considering product yields and reaction rates.

4.1. Enzymatic peptide oligomerization reactions

Oligopeptide products are important components or precursors of pharmaceuticals, medical care products, food or cosmetics (Gill *et al.* 1996; Benns *et al.* 2000; Fairman and Akerfeldt 2005; Deming 2007; Guzman *et al.* 2007; Lupo and Cole 2007; Giessen and Marahiel 2012). These products may be synthesized by chemical or enzymatic routes. Chemical synthesis methods are summarized under SPPS (Solid Phase Peptide Synthesis), LPPS (Liquid Phase Peptide Synthesis) or ROP (Ring Opening Polymerization). The chemical synthesis runs under usage of toxic reagents and organic solvents. For example SPPS and LPPS require repeated protection/deprotection cycles leading to low yields and slow conversion. ROP often requires high temperatures in order to thermodynamically favor the reaction (Myers *et al.* 2002; Opstal *et al.* 2003). Enzymatic synthesis of oligopeptides offers several advantages over chemical reaction as it is conducted under mild reaction conditions and features regio- and enantioselectivity. Protease-catalyzed oligomerization reactions are prominently known from the literature and industrial applications (Morihara *et al.* 1983; Quaedflieg *et al.* 2003; Viswanathan *et al.* 2010; Qin *et al.* 2011). The enzymes are chosen according to protease specificity for certain amino acids acting as reactants. As the natural role of proteases is the hydrolysis of peptides, the hydrolysis pathway is usually being preferred in contrast to synthesis reactions such as amidation of the carboxyl terminus of the substrate. Therefore, an interesting investigation would be exploring the conditions under which the peptide synthesis (i.e. amidation) is more preferred than hydrolysis. An amino acid monomer may be used to generate homooligopeptides. Also amino acid mixtures may be used for rendering heterooligopeptides (Li *et al.* 2008; Qin *et al.* 2013; Andre *et al.* 2014). However, the product composition from these reactions in one-pot reactions is of limited control. A sequential synthesis approach may lead to controlled oligopeptide compositions for certain applications. Furthermore, considerations on product kinetics may provide a better control of by-product formation during reactions leading to maximum synthesis product yields.

Using immobilized enzymes contributes to a better separability and controllability of such reactions and allows for feasibility of more than one-pot reactions. Also, as the immobilization procedure may change the intrinsic characteristics of enzyme, and as a result, different reaction specificities may

occur depending on immobilization conditions (Mateo *et al.* 2007; Rodrigues *et al.* 2012).

4.2. Particle size investigations

As explained in detail in Chapter 1, the particle size of a carrier used for immobilization is one of the most crucial factors for catalyst performance. Investigations on particle size-dependent synthesis or hydrolysis reactions of immobilized enzymes have been addressed in previous publications. Among these works an influence of particle size on the specific activity (Sears and Clark 1993; Barros *et al.* 1998; He *et al.* 2003; Chen *et al.* 2008), substrate specificity (Pencreach *et al.* 1997; He *et al.* 2003), and protein structure (Vertegel *et al.* 2004) of hydrolases in synthesis and hydrolysis reactions was assessed.

The aim of the work presented in this chapter was to develop a kinetically controlled peptide oligomerization reaction as a model reaction in order to observe and investigate the specific activity and product yields of trypsin immobilized on particles of different size. These investigations may give indications of how product formation rates of a sequential reaction change in an enzyme reactor with immobilized biocatalyst. In particular, as trypsin may exhibit two reaction routes, amidation (named synthesis in this section) and hydrolysis, the effect of immobilization on both types of reactions, may be observed in these results.

4.3. Materials and Methods

Magnetic silica particles (Magprep) were provided by Merck KGaA, Darmstadt, Germany. Magnetic agarose (Mag-Trypsin) with immobilized bovine trypsin were purchased from Takara Clontech, Saint-Germain-en-Laye, France. Poly(methacrylate) (PMA) beads with functional amino groups (Relizyme EA 403/S) were purchased from Resindion, Binasco, Italy. Magnetic poly(vinyl alcohol) micro-particles (M-PVA) C22–C250 were provided by PerkinElmer Chemagen, Baesweiler, Germany. 3-Aminopropyltrimethoxysilane (APTMS) and carboxyethylsilanetriol (CEST) were obtained from ABCR GmbH, Karlsruhe, Germany. L-Arginine ethyl ester (Arg-OEt), L-arginine (Arg), L-lysine ethyl ester (Lys-OEt) and L-lysine (Lys) were purchased from Bachem, Weil am Rhein, Germany. All other chemicals originate from sources given in Chapter 2 and 3.

4.3.1. Trypsin-catalyzed oligomerization reaction of arginine and lysine

Oligomer synthesis was conducted with Nα-Benzoyl-L-arginine ethyl ester as activated acyl donor and Arg-OEt or Lys-OEt as nucleophile. Bz-Arg-OEt was used at 10 mM or 20 mM concentration, Arg-OEt/Lys-OEt at 200 mM or 400 mM concentration in 0.5 M Tris buffer pH 8. The reaction was initiated by introduction of immobilized or free trypsin solution at 20 °C under constant shaking. Samples were taken and mixed at a 1:4 (v/v) ratio with 6% TFA in order to terminate the reaction. After a centrifugation step of 1 min at 13 000 rpm, samples were analyzed by HPLC.

4.3.2. HPLC analysis of peptide synthesis products

Peptide synthesis samples were analyzed by RP-HPLC-DAD in a gradient-based method. Eluent A: H_2O and Eluent B: ACN solutions containing 0.1% TFA were used as mobile phase at 1 ml/min flow rate. For oligopeptide analysis, the sample separation started with 5-20% B from 0-14 min, 20-40% B from 14-16 min, and ended with 40-5% from 16-19 min. The method duration was 26 min. The column temperature amounted to 60 °C and the absorbance was measured by a DAD detector at 200 and 254 nm. As standard substances, Arg-/Lys-OEt, Bz-Arg-OEt, and Bz-Arg were used in order to quantify the product concentrations.

4.3.3. MALDI-ToF/MS analysis of oligomerization components

MALDI-ToF/MS analysis was performed on a 4800 Proteomics Analyzer device from Applied Biosystems, Foster City, CA, USA in positive ion linear mode and at a mass range of 130 to 800 Da. The laser intensity was set to 2700. The spectra obtained represent the average of 500 laser shots taken by an automatic scheme measuring spectra over the whole spot. Each sample was mixed at a 1:1 (v/v) ratio with a 10 mg/ml α-cyano-4-hydroxy cinnamic acid (CHCA) in 50 % ACN with 0.1 % TFA and spotted in a duplicate.

4.3.4. Immobilization of trypsin on PMA particles

For immobilization of trypsin onto PMA particles, a 25 mg/ml particle suspension was washed with 1 M potassium phosphate (KPP) buffer pH 7 for three times by alternating suspension and centrifugation steps. A

25% (v/v) GDA solution in 1 M KPP pH 7 was used for activation. The particles were incubated for activation for 1 h at RT. Subsequently, they were washed with KPP buffer for three times and incubated with trypsin as a 0.025 $g_{protein}/g_{particle}$ suspension for 2 h at RT. After the protein coupling step, the particles were washed with 0.040 M Tris buffer pH 9 for three times and the supernatants were used for bound-protein quantification as given in Section 2.3.3 in Chapter 2. Immobilizates were stored at 4 °C in 0.02 M $CaCl_2$.

4.3.5. Amino functionalization of magnetic silica particles

Per g particle, 50 ml EtOH, 80 ml dH_2O, and 3 ml 25% NH_3 were added and mixed. Every 20 min, 2 ml of 96% APTMS solution were added for four times, the total volume of APTMS was 8 ml. The mixture was incubated at RT for 1 h. After incubation, the particles were washed with dH_2O for four times.

4.3.6. Carboxyl functionalization of magnetic silica particles

Magnetic silica particles (Magprep) of 25 and 100 nm were functionalized according to a standard protocol (Hermanson 2008). In brief, a particle suspension of 10 mg/ml was washed for three times with 10 mM sodium phosphate (NaPP) buffer pH 7.4. Subsequently, 2 $ml/g_{particle}$ 25% (v/v) CEST solution in water were introduced to a 10 mg/ml particle suspension and incubated for 4 h at RT. After incubation, the particles were washed with 10 mM NaPP buffer for 5 times and stored in the same buffer at RT.

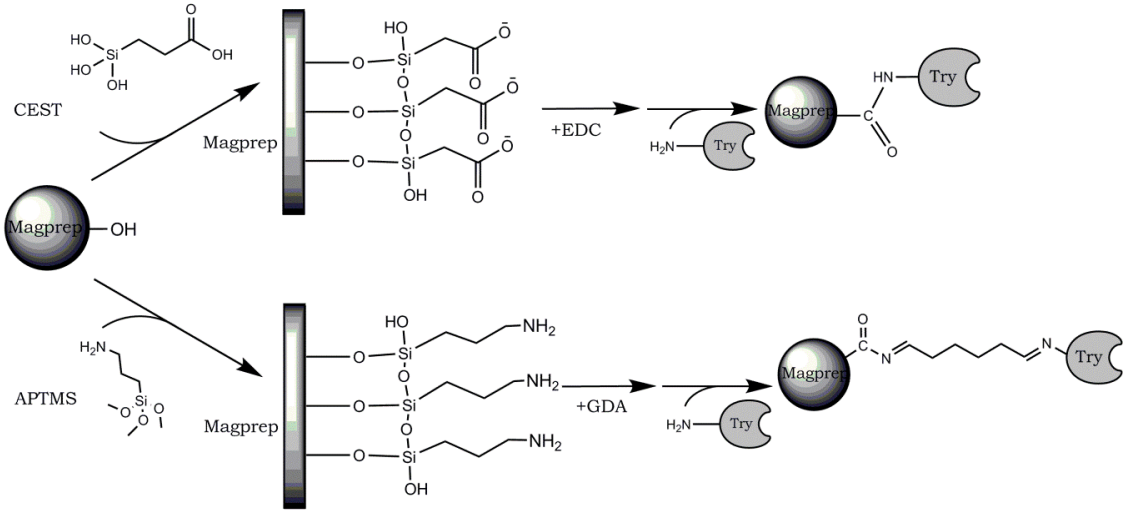
4.3.7. Immobilization onto magnetic particles

For immobilization onto carboxyl-functionalized Magprep particles, a 10 mg/ml particle suspension was adjusted to a 25 mg/ml suspension by magnetization and supernatant exchange with 0.1 M MES buffer pH 5.3. Subsequently, Magprep and M-PVA particles were washed by the same buffer for three times. Furthermore, immobilization was conducted in MES buffer pH 7.1 as described in Section 2.3.1 in Chapter 2.

Amino-functionalized Magprep particles were adjusted to a particle concentration of 25 mg/ml and washed with 0.1 M MES buffer pH 7.1 for three times. A 25% GDA solution in 0.1 M MES pH 7.1 was used for particle activation and was incubated with the particles for 1 h. After the activation step, the particles were washed with the same buffer for three times and incubated with trypsin solution in 0.1 M MES pH 7.1 for 3 h. The washing and

storage steps were conducted as described in Section 2.3.1 in Chapter 2. For all immobilization methods, a 0.015 $g_{protein}/g_{particle}$ input concentration was used.

The functionalization and immobilization procedures with Magprep particles are depicted in Scheme 4.1.



Scheme 4.1. Preparation of Magprep particles with carboxyl, and amino functionalization and subsequent covalent binding of trypsin.

As response factors for free and immobilized enzyme examined in this section, the specific activity and maximum product yield were applied.

$$\text{specific activity} = \frac{U}{g_{protein}} = \frac{\mu mol/min}{g_{protein}} \text{ or } \frac{mAU \times s/min}{g_{protein}}. \quad (4\text{-}1)$$

$$\text{maximum product yield} = 100\% \times \frac{\text{maximum peak area substance}_{t=x}}{\text{substrate peak area}_{t=0}}. \quad (4\text{-}2)$$

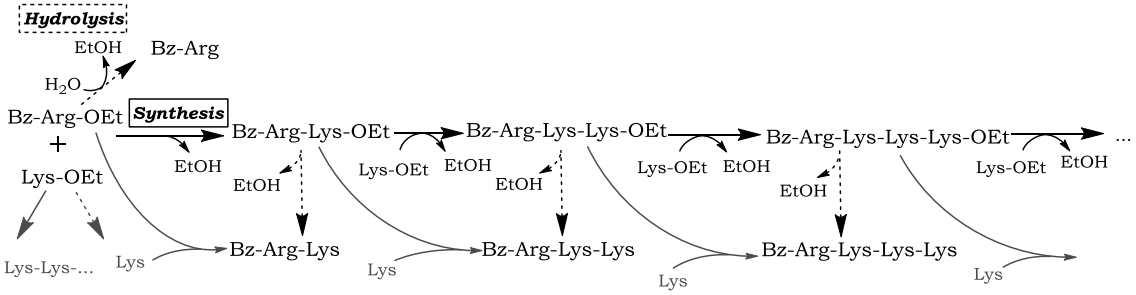
4.4. Results and Discussion

4.4.1. Establishing an oligomerization reaction for trypsin

4.4.1.1. Reaction set-up for oligomerization

As trypsin is a serine protease with specificity for arginine and lysine, a kinetically controlled oligomer synthesis approach in aqueous solution was selected with Bz-Arg-OEt and Lys-/Arg-OEt as reactants (Bongers and Heimer 1994). The theoretical reaction sequence of part-reactions occurring during conversion of Bz-Arg-OEt and Lys-OEt to Bz-Arg-$(Lys)_n$ oligopeptides is depicted in Scheme 4.2. The conversion of Bz-Arg-OEt and Arg-OEt to Bz-Arg-

$(Arg)_n$ is believed to occur in a comparable way. The oligomer synthesis is initiated by binding of the enzyme to Bz-Arg-OEt forming an enzyme-substrate complex intermediate (acyl-enzyme). The reaction is progressed by deacetylation of the enzyme reacting with a nucleophile such as Lys-OEt or water. The reaction with Lys-OEt yields a heterodimer, which is readily activated for oligomerization propagation. The reaction with water results in a hydrolysis of the Bz-Arg-OEt to Bz-Arg and ethanol. As Lys-OEt may also act as a potential substrate, the oligomerization to oligo(Lys) may not be ruled out. However, in this study the targeted reaction was Bz-Arg-OEt-initiated oligomerization reaction.



Scheme 4.2. Reaction scheme of Bz-Arg-OEt elongation by Lys-OEt monomers to oligomers with side reactions. Synthesis steps are indicated with solid arrows, hydrolysis steps are shown with dashed arrows.

4.4.1.2. Identification of oligomerization products via MALDI-ToF/MS

Reactants and products from both trypsin-catalyzed oligomerization reactions Bz-Arg-OEt + Arg-OEt and Lys-OEt were separated via the HPLC method for identification and further quantitation. Reactant fractions from the HPLC separation method were collected and MALDI-ToF/MS analysis was performed for the collected samples. Mass spectra of collected fractions are given in Table B.1 of supplemental material. A comparison of the predicted and resulting molecular weights of reactants was accomplished. Table 4.1 shows the results of the measurement. Five reactant peaks within Bz-Arg-OEt + Arg-OEt reaction and eleven within Bz-Arg-OEt + Lys-OEt could be explicitly identified via mass determination. Typical chromatograms from sample separation via HPLC for both reactions are shown in Figure 4.1. In this respective chromatogram sample, the absorbance was measured at 254 nm depicting substances with benzoyl residues (Bz-). The numbered peaks are marked and referred to products in Table 4.1. From samples, oligopeptides of several different polymerization grades were verified. Within the benzoyl esters (Bz-X-

OEt), di- and tripeptides were detected. The substances containing a benzoyl residue with an up to tetrapeptide stage were identified.

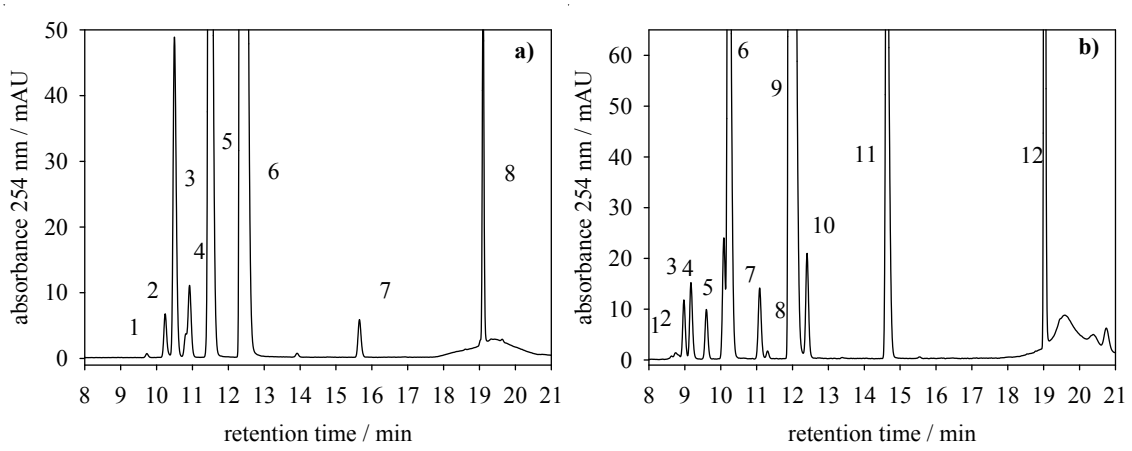


Figure 4.1. Chromatograms for reactant separation via HPLC and peak numeration of substances obtained from reaction a) Bz-Arg-OEt + Arg-OEt and b) Bz-Arg-OEt + Lys-OEt.

Table 4.1. Peak identification from different peptide oligomerization reactions by MALDI-ToF/MS.

	Bz-Arg-OEt + Arg-OEt			Bz-Arg-OEt + Lys-OEt		
Peak number	retention time	found mass / Da	substance	retention time	found mass / Da	substance
1	9.8	-	-	8.60	-	-
2	10.2	-	-	8.74	662.65	Bz-Arg-Lys-Lys-Lys
3	10.5	-	-	8.98	534.70	Bz-Arg-Lys-Lys
4	10.9	590.65	Bz-Arg-Arg-Arg	9.17	534.69	Bz-Arg-Lys-Lys
5	11.5	434.65	Bz-Arg-Arg	10.09	406.74	Bz-Arg-Lys
6	12.40	278.76	Bz-Arg	10.22	406.74	Bz-Arg-Lys
7	15.70	462.71	Bz-Arg-Arg-OEt	11.09	278.73	Bz-Arg
8	19.10	306.78	Bz-Arg-OEt	11.30	-	-
9				11.93	278.73	Bz-Arg
10				12.40	434.79	Bz-Arg-Lys-Lys-OEt
11				14.63	434.79	Bz-Arg-Lys-OEt
12				19.03	306.78	Bz-Arg-OEt

4.4.1.3. Optimization of oligomerization reaction parameters

For conduction of peptide oligomerization reaction at optimum conditions, several parameters optimized for dimerization reaction from Chapter 2 were adopted. An optimum reaction temperature of 20 °C and a pH of 8 were applied. Furthermore, the substrate ratios as well as water content for oligomerization reaction were specifically optimized in this part. Figure 4.2 shows the resulting optimal values for substrate ratio and water content in the oligomerization medium. As the oligomerization reaction is based on a kinetically controlled approach, the reaction rate is at its highest at 100% of water in the medium (Figure 4.2 c). Varying the substrate ratio, the reaction rate of the dimer ester Bz-Arg-Arg/Lys-OEt is higher than the resulting dimer hydrolysis product Bz-Arg-Arg/Lys (Figure 4.2 a, b) at a molar excess of Bz-Arg-OEt and equimolar substrate ratio.

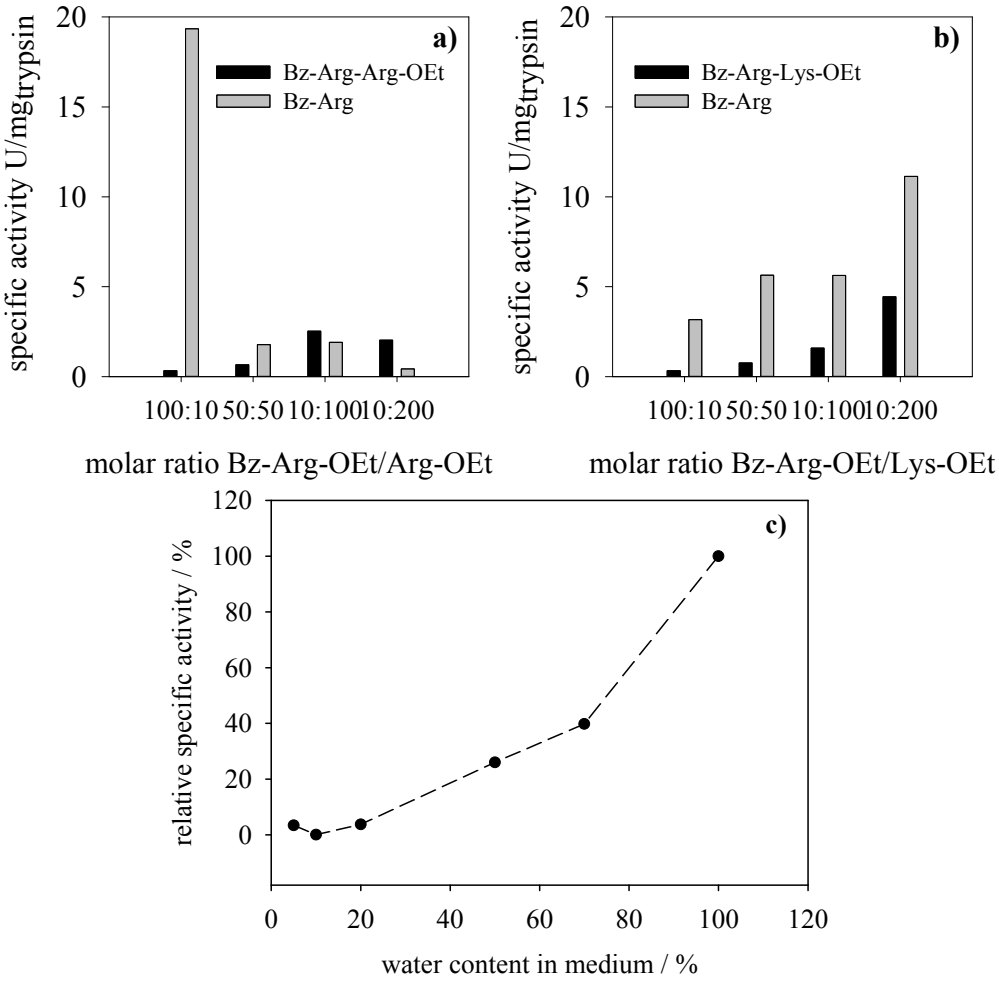


Figure 4.2. Specific activity of trypsin for hydrolysis product formation Bz-Arg and synthesis product formation Bz-Arg-Arg and Bz-Arg-Lys in peptide oligomerization reaction with varying substrate ratios in a) Bz-Arg-OEt + Arg-OEt and b) Bz-Arg-OEt + Lys-OEt; c) specific activity of trypsin relative to the maximum specific activity achieved in this experiment varying water content in reaction medium for reaction of Bz-Arg-OEt + Arg-OEt and Bz-Arg-Arg formation.

However, the product formation rate of Bz-Arg-Arg/Lys-OEt increases as the molar concentration of the nucleophile (Lys/Arg-OEt) exceeds Bz-Arg-OEt. A possible explanation is the concentration of the nucleophile Lys/Arg-OEt. Schellenberger and Jakubke introduced a partition constant p (Equation 4-3) for kinetically controlled enzymatic peptide synthesis reactions (Schellenberger and Jakubke 1991). In analogy to the Michaelis-Menten constant, this value corresponds to the nucleophile concentration at which aminolysis and hydrolysis of the acyl-enzyme proceed with the same velocity at the time when the substrate ester is completely converted.

$$p = \frac{[\text{hydrolysis product}]}{[\text{synthesis product}]} \times [\text{nucleophile}]. \qquad (4\text{-}3)$$

Consequently, the concentration of the nucleophile must exceed the p-value in order to provide sufficient synthesis product yields. The variation in the p-constants of different nucleophiles reveals the efficiency of a certain nucleophile for S1' subsite binding of the respective enzyme. Table 4.2 shows the calculated p-values for this reaction with Arg/Lys/Arg-/Lys-OEt acting as nucleophiles. The hydrolysis product concentration was related to the sum of all synthesis products at a given nucleophile concentration. For Lys and Lys-OEt, the p-value is higher than for Arg and Arg-OEt, which means that higher concentrations of the first are needed in order to obtain higher synthesis than hydrolysis reaction rates. Binding of Arg-X based nucleophiles in S1' position is more favored compared to Lys-X based nucleophiles. This is supported by the comparison of hydrolysis and synthesis rates in Figure 4.2 for Arg- (a) and Lys-OEt (b). While the synthesis-specific activity of trypsin increases with the nucleophile/substrate ratio of >10 mM:100 mM using Arg-OEt and thus exceeding the hydrolysis, the synthesis activity using Lys-OEt remains slower compared to the hydrolysis reaction rate even at a 10 mM:200 mM ratio. Similar findings were made by Hanisch *et al.* (1987). The partition constant of arginine based nucleophiles was higher than the constant for lysine-nucleophiles. Trypsin shows a preference for arginine residues binding to the S1' position of trypsin, whereas lysine binding is less favored (Hanisch et al. 1987). Furthermore, a preference for nucleophiles with ester groups could be observed comparing the p-constant of the amino acids Arg and Lys to their corresponding esters. Presumably, a longer-chained nucleophile with less polar C-terminus is better accepted by the substrate-binding site of trypsin.

Table 4.2. Partition constant of Bz-Arg-OEt conversion with nucleophile.

Nucleophile	Arg	Arg-OEt	Lys	Lys-OEt
Bz-Arg-OEt : nucleophile conc. /mM	10:200	10:200	10:200	10:200
p-constant/mM	283.69	176.32	1066.7	263.56

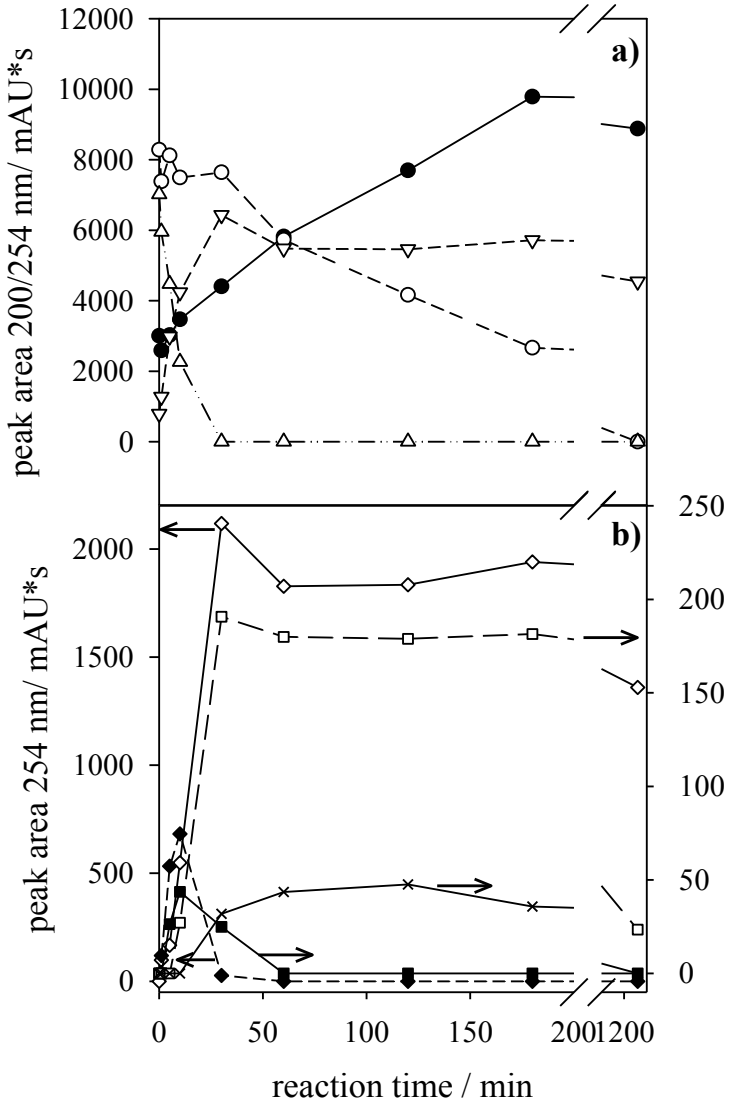


Figure 4.3. Time course of substrate conversion and product formation in trypsin-catalyzed oligomerization reaction. Reactants are coded as follows: a) Bz-Arg-OEt △, Bz-Arg, Lys-OEt ○, Lys ●; b) Bz-Arg-Lys-OEt ◆, Bz-Arg-Lys ◇, Bz-Arg-Lys-Lys-OEt ■, Bz-Arg-Lys-Lys □, Bz-Arg-Lys-Lys-Lys ×. Arrows refer plotted data to respective ordinate.

4.4.1.4. Product kinetics of free trypsin

At starting conditions of 10 mM Bz-Arg-OEt and 200 mM Lys-OEt, the product kinetics of a free trypsin-catalyzed oligopeptide reaction were monitored. Figure 4.3 shows the product kinetics of free trypsin. Whereas Lys-OEt and Lys concentrations are given as absorbance values at 200 nm, all remaining products are measured at 254 nm in order to relate the formed amounts of

substances. In Figure 4.3 a) it is shown how the substrates Bz-Arg-OEt and Lys-OEt are converted to Bz-Arg and Lys. The total hydrolysis of Bz-Arg-OEt occurs within 30 min after the reaction starts, whereas Lys-OEt is fully converted after 21 hours. Lys-OEt is mostly hydrolyzed to Lys by 70%, 30 % are used for Bz-Arg-Lys-OEt synthesis. Bz-Arg-OEt is hydrolyzed to Bz-Arg up to 53%, whereas about 47% yield sequential synthesis products, mostly Bz-Arg-Lys-OEt and Bz-Arg-Lys. From Scheme 4.2, it may be derived that sequential product formation steps result in the accumulation of peptide esters (-OEt) as intermediates, from which more stable hydrolysis end products may be formed. In Figure 4.3 b), kinetics of Bz-residue products synthesis and their respective hydrolysis products are depicted. After 1 min of reaction time, Bz-Arg and Bz-Arg-Lys-OEt are built. After 5 min of reaction time, Bz-Arg-Lys and Bz-Arg-Lys-Lys-OEt are derived from the latter. The maximum concentration of Bz-Arg-Lys-OEt, as well as Bz-Arg-Lys-Lys-OEt, is reached by 10 min and lagged by 20 min (at 30 min), the maximum of Bz-Arg-Lys and Bz-Arg-Lys-Lys may be observed. Bz-Arg-Lys-Lys-Lys, a tetrapeptide, is measurable after 30 min showing a maximum at 120 min. The collected experimental data support the reaction course depicted in Scheme 4.2.

4.4.2. Comparison of product formation kinetics and profiles of different particle species

The characterized oligomerization reaction may serve as model reaction for comparison of product formation kinetics, specificity and mass diffusion characterization of several carriers. Particle species of different sizes and surface characteristics were used for immobilization of trypsin. Mag-Trypsin with immobilized bovine trypsin was commercially available. The characteristics of the used particles are listed in Table 4.3.

Table 4.3. Particle characteristics of carriers used in this chapter.

Particle type	R/nm	porous/non-porous
free trypsin	3	-
Magprep	25	non-porous
Magprep	100	non-porous
M-PVA	1000	non-porous
Mag-Trypsin	50000	porous
PMA	100000	porous

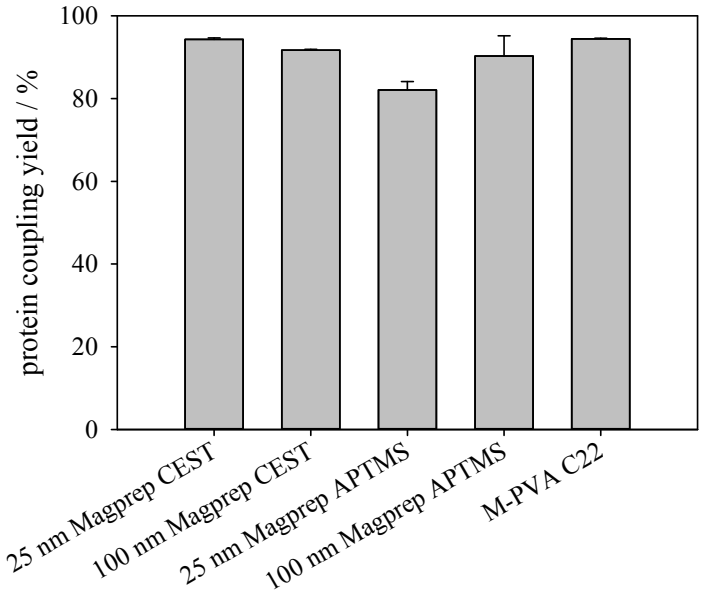


Figure 4.4. Protein coupling yields for covalent trypsin coupling onto carboxyl (CEST) and amino (APTMS) functionalized magnetic particles.

Magprep particles were first functionalized with carboxyl or amino groups using two different methods. The resulting protein coupling yields showed the CEST-based functionalization as best suitable for trypsin binding (Figure 4.4). These particles were used for further investigations. After the immobilization procedure, the resulting activity was measured in a dipeptide synthesis reaction used in Chapters 2 and 3.

In order to compare the product kinetics and product profiles of these immobilizates in oligomerization reaction, the same activities calculated from dipeptide synthesis assay for each immobilizate were applied. The concentration of Bz-Arg-OEt amounted 20 mM, while Lys-OEt was used as nucleophile at 400 mM in this section. Other reaction conditions were identical to thos described in previous sections. The resulting specific activities for hydrolysis and synthesis product formations from peak area values in mAU*s (Equation 4-1) are depicted in Figure 4.5 as a function of particle size. Although the same immobilizate activities from dipeptide synthesis reaction Bz-Arg-OEt+Arg-NH_2 were introduced in the oligomerization reaction, markedly different specific activities for these immobilizates were observed. The specific activities for hydrolysis and synthesis products decrease with increasing particles sizes. In the hydrolysis reaction of Bz-Arg-OEt to Bz-Arg, particle size increase shows the highest decrease in activity. Mag-Trypsin particles with $5x10^4$ nm deviate from the general trend shown in the specific activity courses. This deviation may result from the protein loading determination on these particles. As the actual protein loading of these

particles was not given by the manufacturer a destructive method for protein extraction from particle polymer was applied. In detail, the $5x10^4$ nm Mag-Trypsin particles were incubated in 0.8 M acetic acid pH 3.3 at 30 °C for 19 h. After centrifugation for 1 min at 13000 rpm the supernatant was used for BC protein quantification assay as given in Materials and Methods section. The resulting protein concentration was applied to calculate the specific activity of the particles in oligomerization reaction. As it may not be ruled out that the protein extraction from the particles was incomplete and the actual protein loading of Mag-Trypsin particles is higher than measured, the resulting specific activity may be lower than calculated from protein determination method.

The specific activity of free enzyme for hydrolysis of Bz-Arg-OEt is 30-fold higher than for Bz-Arg-Lys-OEt synthesis. Accordingly, the mass transport limitation may occur more significantly in the hydrolysis reaction step than in the synthesis reaction. During the synthesis of Bz-Arg-Lys-OEt and in the sum of all synthesis activities of different oligomers from this reaction, the particle size becomes a significant influence factor when exceeding 100 nm. Immobilizates with particle sizes below 100 nm exhibit specific activity values comparable to free enzyme. Due to lower reaction rates in synthesis route, mass transport limitation is less significant using small non-porous particles. From the specific activity measurements, no distinction of porous and non-porous particle species or chemical particle properties in hydrolysis or synthesis route may be made. The particle size is experimentally shown to occur as a crucial impact factor in the specific activity of immobilized trypsin in hydrolysis and synthesis reactions. However, more detailed considerations on the ratio of hydrolysis to synthesis rates may be an interesting response factor giving insights into process-related reaction route control.

The ratio of synthesis/hydrolysis activity for all tested particle species was calculated from Equation 4-4 and is depicted in Figure 4.6 as a function of particle size.

$$\text{ratio synthesis/hydrolysis} = \frac{\sum \text{specific activity}_{\text{synthesis products}}}{\text{specific activity}_{\text{Bz-Arg}}}. \qquad (4\text{-}4)$$

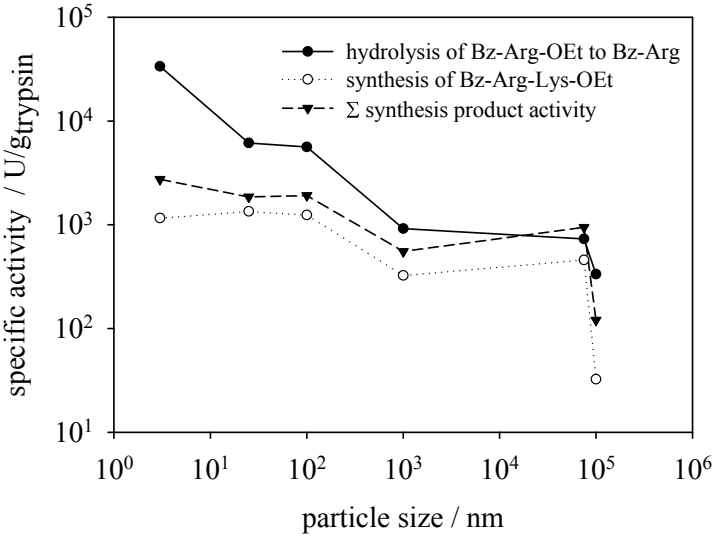


Figure 4.5. Specific activities for trypsin immobilized on particles of different size for different oligomerization product formations.

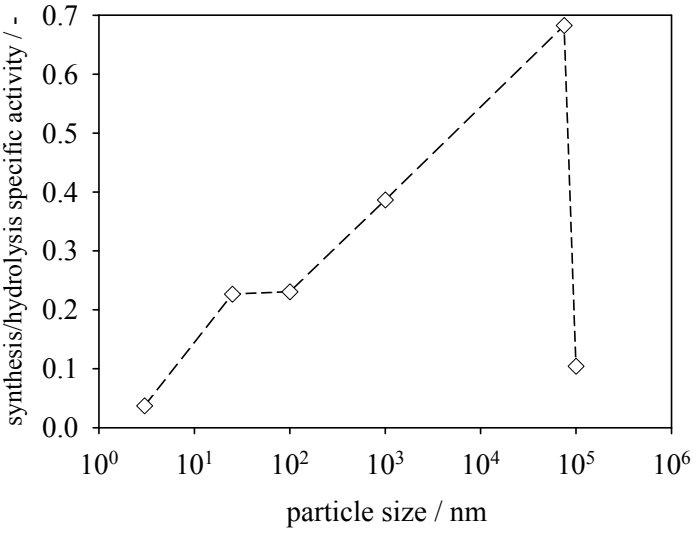


Figure 4.6. Ratio of synthesis to hydrolysis specific activity in oligomerization reaction for trypsin immobilized on different particle species.

The ratio of synthesis-to-hydrolysis activity increases towards higher particle sizes with the exception of porous PMA particles with 10^5 nm. This indicates a limitation of Bz-Arg-OEt transport to the immobilized enzyme leading to slower hydrolysis of Bz-Arg-OEt from bulk medium facilitating the synthesis route with the second reactant Lys-OEt. Results indicating a similar behavior were obtained by Sears and Clark (1993). Trypsin was immobilized on controlled pore glass with 0.45 µm pore size. A comparison of esterase/amidase kinetic parameters and energy diagrams of free and immobilized enzyme lead to an observed raised free energy barrier for amide and ester hydrolysis and lowered energy barrier for aminolysis. The authors stated that immobilization rendered an enzyme more effective in peptide synthesis than in hydrolysis. This was explained by the fact that ester hydrolysis is less dependent on the

surrounding structure of the active site, while amidase activity is more sensitive to conformational changes close to the active site. As immobilization changes the conformation of the enzyme the amidase activity is favored thereby. However, not all effects of immobilization observed in this work could be explained by this theory (Sears and Clark 1993). PMA particles of 10^5 nm represent an exception from the increase of synthesis/hydrolysis ratio as a function of the particle size. The nature of these highly porous particles featuring 60-70% water retention and 40-60 nm pore size (Resindion 2015) may cause an enzyme conformation with less changes surrounding the active site favoring the hydrolysis step over synthesis compared to other carriers. Another unique characteristic of PMA particles compared to other used particle species is the absence of magnetic properties. In previous publications a significant adverse effect of magnetic properties on hydrolytic enzymatic activity of α-chymotrypsin and lipase was observed comparing immobilization on same material with and without magnetite (Chen and Su 2001; Chen and Lin 2003). In these works, the decrease of activity was explained by increased hydrophilicity of the carrier upon magnetite addition thereby negatively influencing enzyme activity. In this work, an adverse effect of magnetic properties of the tested particles may have favored the synthesis reaction upon hydrolysis and caused a variance of trend for non-magnetic PMA particles in Figure 4.6.

In order to examine the limitation of the second reactant Lys-OEt, the afore mentioned p-constant was calculated for all tested particles in oligomer synthesis reaction. Comparable to the dimensionless substrate concentration β from Chapter 1, the p-value calculated for this reaction was modified by relating the value to the bulk nucleophile concentration at t=0. The normalization facilitates a direct conclusion about nucleophile excess and synthesis/hydrolysis rate ratio.

$$\text{normalized p} = \frac{[\text{nucleophile}]_{t=0}}{\frac{[\text{hydrolysis product}]}{[\text{synthesis product}]} \times [\text{nucleophile}]}. \tag{4-5}$$

Table 4.4 gives a comparison of normalized p-coefficients from Equation 4-5 at oligomerization conditions for all tested carriers. The calculated normalized p-coefficients are found to be lower than or equal to the used nucleophile concentration of 400 mM with the exception of free enzyme and PMA particles suggesting reaction with an excess of nucleophile for M-PVA and Mag-Trypsin. The p-constant of Magprep particles indicated an equal preference in

hydrolysis and synthesis product formations. Mag-Trypsin shows a slight excess of nucleophile. With M-PVA particles, the highest normalized p-constant of 3.6 was achieved. This fact indicates that immobilization on M-PVA particles renders a more effective synthesis compared to the free enzyme. This increase of p may be a result of diffusion limitation of Bz-Arg-OEt to the immobilized enzyme on M-PVA. For a dimerization of substrates, Bz-Arg-OEt and Lys-OEt both must be bound in the active site of the enzyme. Lys-OEt features a higher molar concentration in bulk reaction medium, and hydrolysis of Bz-Arg-OEt is suppressed since Lys-OEt may bind first with a higher substrate transport gradient to the particle surface. Resulting from this theory Bz-Arg-Lys may be synthesized from two reactions: Bz-Arg-Lys-OEt hydrolysis and synthesis with Bz-Arg-OEt + Lys (Scheme 4.2). Further considerations of the maximum yields of the respective reaction products must be undertaken.

Table 4.4. Normalized partition constant of Bz-Arg-OEt conversion with nucleophile for particles of different size relating hydrolysis product formation of Bz-Arg to the sum of all synthesis products formed in the reaction.

	free trypsin	Magprep 25 nm	Magprep 100 nm	M-PVA	Mag-Trypsin	PMA
norm. p/-	0.64	1.03	1.03	3.58	1.56	0.82

The maximum product yields were calculated for all detected products according to Equation 4-2 within different particle-catalyzed reactions and depicted in Figure 4.7. The maximum yield of the hydrolysis product Bz-Arg may reach over 80% and is found applying porous PMA particles as catalyst, which is higher than free enzyme showing a 73% hydrolysis product yield. The lowest Bz-Arg yield of 49% was observed within non-porous M-PVA particles. The first synthesis product Bz-Arg-Lys-OEt may reach a maximum yield of 18% with M-PVA particles and 14.4% with Mag-Trypsin as second highest. The resulting hydrolysis product Bz-Arg-Lys shows a yield of 42% which is equally measured within 10^3 nm M-PVA and $5x10^4$ nm Mag-Trypsin particles. With free enzyme only 31% and with 10^5 nm PMA particles, 22% Bz-Arg-Lys may be reached. Considering further sequential synthesis products, M-PVA particles show the highest yields of tri- and tetrapeptides Bz-Arg-Lys-Lys-OEt, Bz-Arg-Lys-Lys and Bz-Arg-Lys-Lys-Lys among all tested particle sizes. From these results, it may be concluded that porous 10^5 nm PMA particles provide a higher hydrolysis product yield, which is above free enzyme. Trypsin

immobilized on these particles is suitable for lowering by-product formation during enzymatic peptide hydrolysis reactions. Presumably, the porous nature and low conversion rates within 10^5 nm PMA particles (Figure 4.5) may provide for higher hydrolysis product yield due to local substrate accumulation in carrier pores. This is supported by the lowest specific activity value and the highest hydrolysis product yield of these particles exceeding the maximum hydrolysis yield value of the free enzyme. On the other hand, non-porous 10^3 nm M-PVA particles feature highest synthesis product yields and appear to be better applicable to peptide synthesis reactions. Unlike the specific activity, the maximum yield of a certain reaction product features an optimum behavior as a function of particle size. Given a kinetically controlled product modification or oligomerization reactions, the process may become better controllable by varying the particle size of the immobilizate and thus achieving a higher yield of a desired oligomer degree.

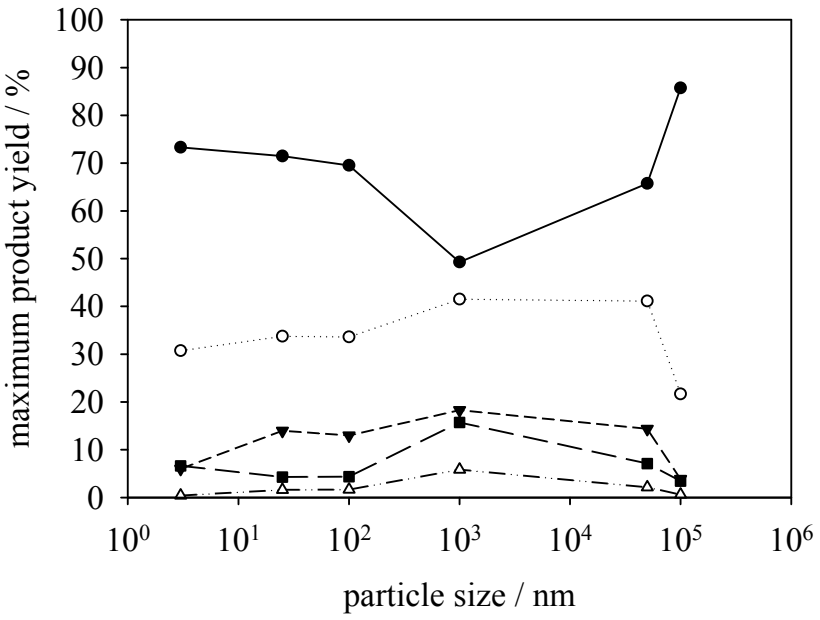


Figure 4.7. Maximum yields of products ● Bz-Arg, ○ Bz-Arg-Lys, ▼ Bz-Arg-Lys-OEt, △ Bz-Arg-Lys-Lys-OEt, ■ Bz-Arg-Lys-Lys from oligomerization reaction with different particles species.

4.5. Summary of Chapter 4

In this chapter, a model reaction for trypsin-catalyzed peptide oligomerization was implemented and characterized. The oligomerization reaction contains peptide hydrolysis and synthesis steps giving an interesting model system for reaction rates, products yields, and by-product formation. Using this model reaction trypsin immobilizates of different size were subjected to a comprehensive comparison. A significant impact of particle size on the specific activity in peptide hydrolysis and synthesis reactions was detected, which may be explained by mass transport limitation of the first substrate to the bound enzyme. Considering the synthesis/hydrolysis ratio of different particle species, a shift towards the synthesis activity with increasing particle size could be detected which may be attributed to conformational changes of the enzyme upon immobilization. Furthermore, M-PVA particles of 10^3 nm have achieved highest yields of synthesis products and thus may be characterized as being best suitable for synthesis product formation, whereas large porous PMA particles of 10^5 nm feature highest hydrolysis product yields. These findings offer an additional tool for industrial yield and conversion rate control by biocatalyst immobilization and carrier choice.

5. Concluding remarks

Industrial enzyme application for fine chemicals and pharmaceuticals production often affords ways for separation and reuse of the enzyme in the biotransformation process. A suitable technique to enable these steps is enzyme immobilization on a non-soluble carrier. However, these techniques may alter the physical and catalytic characteristics of the biocatalyst and must therefore be tailored individually depending on the enzymes' application. In this study trypsin a protein cleaving enzyme, which is commonly applied for industrial peptide hydrolysis and synthesis processes, was used as a model enzyme for immobilization. A holistic approach for process driven enzyme immobilization optimization by method, particle surface and size considerations was successfully implemented. A dipeptide synthesis reaction was implemented as model for synthesis activity assessment after trypsin immobilization. In particular, a new immobilization method in organic medium was developed and compared to a standard method in aqueous medium. The immobilization methods featured different impacts on hydrolysis and synthesis activities. Organic immobilization achieved up to sixfold higher synthesis activity of immobilized trypsin in comparison to the aqueous method. The hydrolysis activity of trypsin from aqueous immobilization was similar to that from organic solvent. These changes in reaction specificity are explained as a result from immobilization medium. Trypsin immobilized under water-controlled conditions may rigidify the conformation of the last aqueous state, while immobilization under aqueous conditions allows for higher protein flexibility leading to active state loss. Varying the protein loading for immobilization resulted in the conclusion that mass transport limitations occur predominantly during hydrolysis reaction, which features faster reaction rates, but less significantly during synthesis reaction with immobilized trypsin. Trypsin immobilized via both methods showed a good reusability in synthesis and hydrolysis reactions with minor activity loss over ten sequential catalysis cycles. Improved synthesis activity for three of the four tested spacer groups were obtained modifying the particle surface by spacer coupling. Additionally, the increase of spacer length was a significant factor for

synthesis activity improvement. However, a combination of organic immobilization method and spacer application did not achieve an amplifying effect for either synthesis or hydrolysis enzyme activities. Changing the trypsin coupling strategy from covalent to affinity binding resulted in an activity preserving enzyme coupling method with improved synthesis and hydrolysis activities. Investigations on the influence of particle size resulted in the conclusion that particle size increase is the predominant negative impact factor for tryptic activity performance in hydrolysis and synthesis reactions. A model reaction for enzymatic amino acid oligomerization with competing hydrolysis and synthesis routes was developed in order to analyze the synthetic and hydrolytic by-product formation in dependence of particle size. The hydrolysis-to-synthesis-activity ratio decreased with increasing particle size with exception of largest porous particles. Large porous particles are most suitable for one-step peptide hydrolysis reactions. Formation of synthesis by-products is low using these particles. Small and middle-sized non-porous particles render higher yields of synthesis products suppressing the hydrolysis product formation. Enzyme immobilization method and choice of carrier represent new tools for influencing the conversion rates and yields of a specific process.

With findings made in this work crucial influence factors for trypsin activity, reaction specificity, reusability, mass-transport behavior and by-product formation within peptide hydrolysis and synthesis reactions were filtered and influence factor boundaries were covered. Knowing the major influencing factors of immobilization a more complex biocatalytic process may be controlled by these factors. For example heterooligopeptide synthesis in a one-pot-reaction yielding a prime peptide product with a defined amino acid composition may be targeted as a model reaction. Furthermore, a scale-up of the established reactions may give information about feasibility in industrial applications and dimension-related influence factors. Transferring the elaborated methods to a biocatalyst with a different reaction mechanism such as laccase or aldolase may give additional information on method specificity. Immobilization method optimization completed in this work delivers a well separable enzyme optimized for peptide synthesis reactions with good activity and reusability. The implemented dipeptide and oligopeptide synthesis reactions may serve as model reactions for future activity determination on an industrially relevant synthesis route.

Abbreviations and Symbols

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
APTMS	3-aminopropyltrimethoxysilane
Arg	arginine
BAPNA	Nα-Benzoyl-DL-arginine p-nitroanilide
BC	bicinchoninic acid
BSA	bovine serum albumin
Bz-Arg	Nα-Benzoyl-L-arginine
Bz-Arg-OEt	Nα-Benzoyl-L-arginine ethyl ester
CABS	4-(cyclohexylamino)-1-butanesulfonic acid
CAPSO	3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid
CDI	1,1′-carbonyldiimidazole
CEST	carboxyethylsilanetriol
CV	coefficient of variation
DAD	diode array detector
dH2O	deionized water
DSC	disuccinimidyl carbonate
EC	enzyme commission (number)
EDC	1-ethyl-3-(3-domethylaminopropyl)carbodiimide
GDA	glutaraldehyde solution
HPLC	high-performance liquid chromatography
KPP	potassium phosphate buffer
LC-Trypsin	long-chain trypsin
Lys	lysine
MALDI-ToF	matrix-assisted laser desorption/ionization- time of flight
MES	2-(N-morpholino)ethanesulfonic acid
MS	mass spectrometry
MW	molecular weight
NaPP	sodium phosphate buffer
pH	- $\log_{10}$ of H+ concentration
PMA	poly(methacrylate)
pNA	p-nitroaniline
pNPP	p-nitrophenol palmitate
PVA	poly(vinyl alcohol)
RP	reversed phase
rpm	rotations per minute

RT	room temperature
TFA	trifluoracetic acid
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet (absorption detector)
A_s	surface activity of carrier
Bi	Biot number
D	diffusion coefficient
Da	Dalton, molecular weight unit
Da_{II}	Damköhler number second order
k	reaction rate
Km	Michaelis-Menten constant
L	characteristic length
p	partition constant of enzymatic synthesis reaction
P	product
q	protein loading
R	particle radius
Re	Reynolds number
S	substrate
s	substrate concentration
Sc	Schmidt number
t	time
T	temperature
v/v	volume per volume
V_{max}	maximum volumetric enzyme activity
w/v	weight per volume
β	dimensionless substrate concentration
ε	porosity of support
η	effectiveness factor
υ	specific enzyme activity
ρ	wet particle density
τ	tortuosity of pores
Φ	Thiele modulus

Appendix

A Supplemental material Chapter 2

Table A.1. Run table of statistical design for optimization of immobilization method in dioxane

Run	Type	Factor 1 A:cCDI in mg/ml	Factor 2 B:activation time in min	Factor 3 C:coupling time in h
1	Axial	32.50	77.5	1.0
2	Axial	32.50	77.5	1.0
3	Factorial	15.00	35.0	2.0
4	Factorial	15.00	35.0	2.0
5	Factorial	50.00	35.0	2.0
6	Factorial	50.00	35.0	2.0
7	Factorial	15.00	120.0	2.0
8	Factorial	15.00	120.0	2.0
9	Factorial	50.00	120.0	2.0
10	Factorial	50.00	120.0	2.0
11	Axial	32.50	6.0	3.5
12	Axial	32.50	6.0	3.5
13	Axial	3.07	77.5	3.5
14	Axial	3.07	77.5	3.5
15	Center	32.50	77.5	3.5
16	Center	32.50	77.5	3.5
17	Center	32.50	77.5	3.5
18	Axial	61.93	77.5	3.5
19	Axial	61.93	77.5	3.5
20	Axial	32.50	149.0	3.5
21	Axial	32.50	149.0	3.5
22	Factorial	15.00	35.0	5.0
23	Factorial	15.00	35.0	5.0
24	Factorial	50.00	35.0	5.0
25	Factorial	50.00	35.0	5.0
26	Factorial	15.00	120.0	5.0
27	Factorial	15.00	120.0	5.0
28	Factorial	50.00	120.0	5.0
29	Factorial	50.00	120.0	5.0
30	Axial	32.50	77.5	6.0
31	Axial	32.50	77.5	6.0

Table A.2. Constraints set for statistical design optimization of response factors for trypsin immobilization in dioxane.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: cCDI/mg/ml	in range	3	60	1	1	3
B: activationtime/min	in range	6	180	1	1	3
C: coupling time/h	in range	1	7	1	1	3
protein coupling yield/%	maximize	51.37	100	1	1	3
carrier activity/U/$g_{particle}$	maximize	1	9	1	1	3
activity coupling yield/%	maximize	1	30	1	1	4
relative specific activity /%	maximize	10.84	42.49	1	1	4
reusability/%	maximize	70.74	100.3	1	1	5

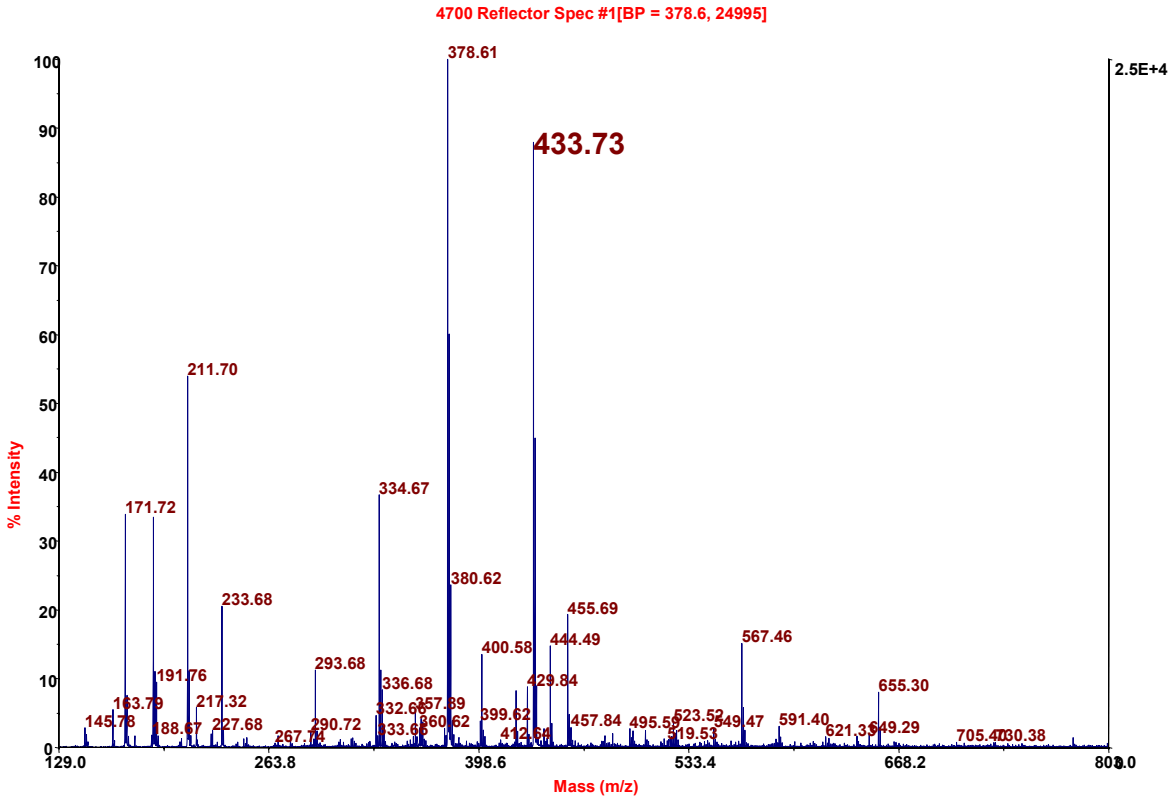


Figure A.1. Mass confirmation of dipeptide product with 433.73 Da via MALDI-ToF/MS

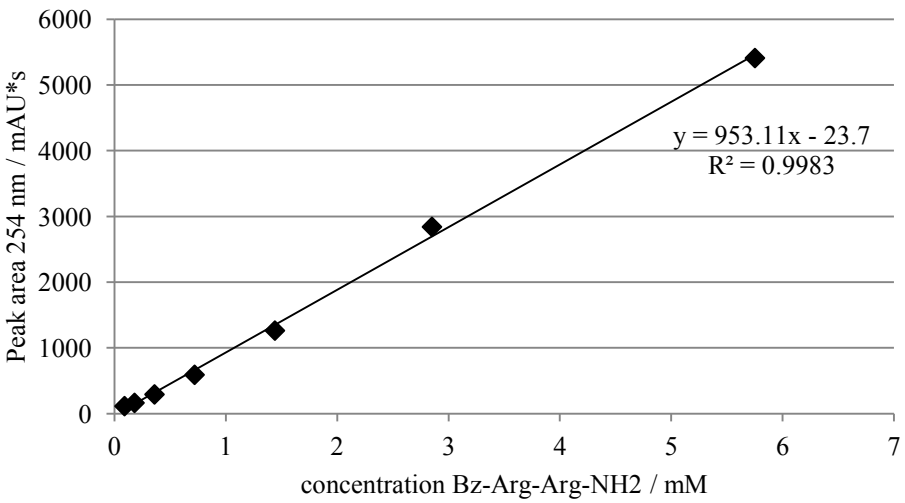
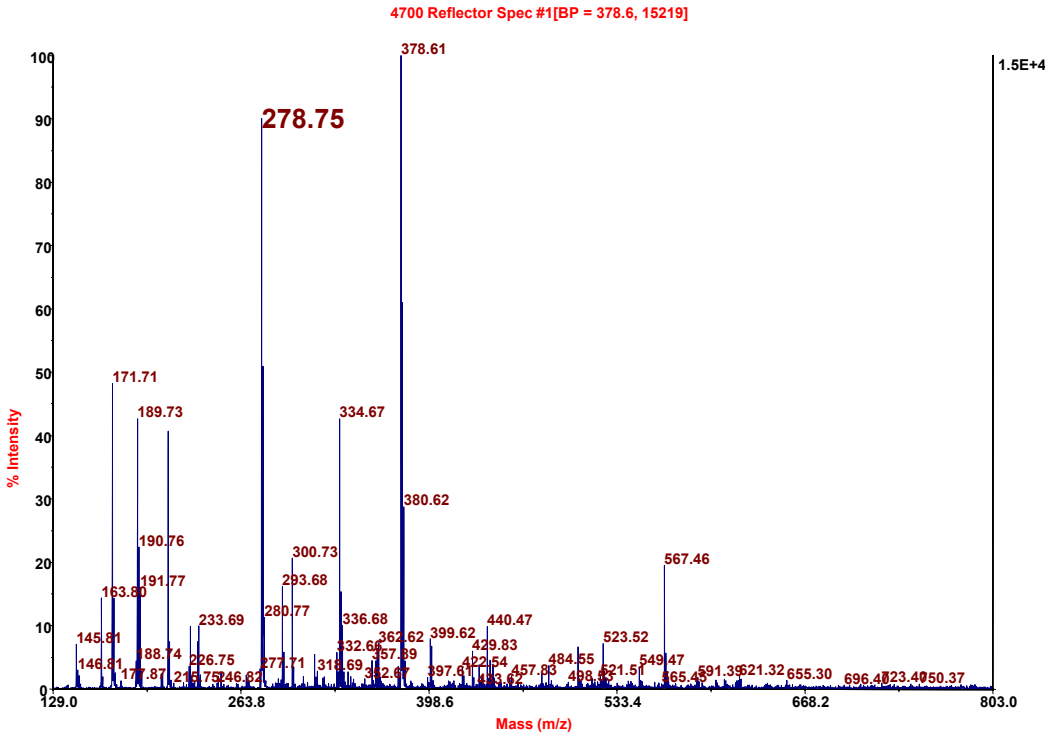


Figure A.2. Calibration of dipeptide product Bz-Arg-Arg-NH_2 in HPLC analysis

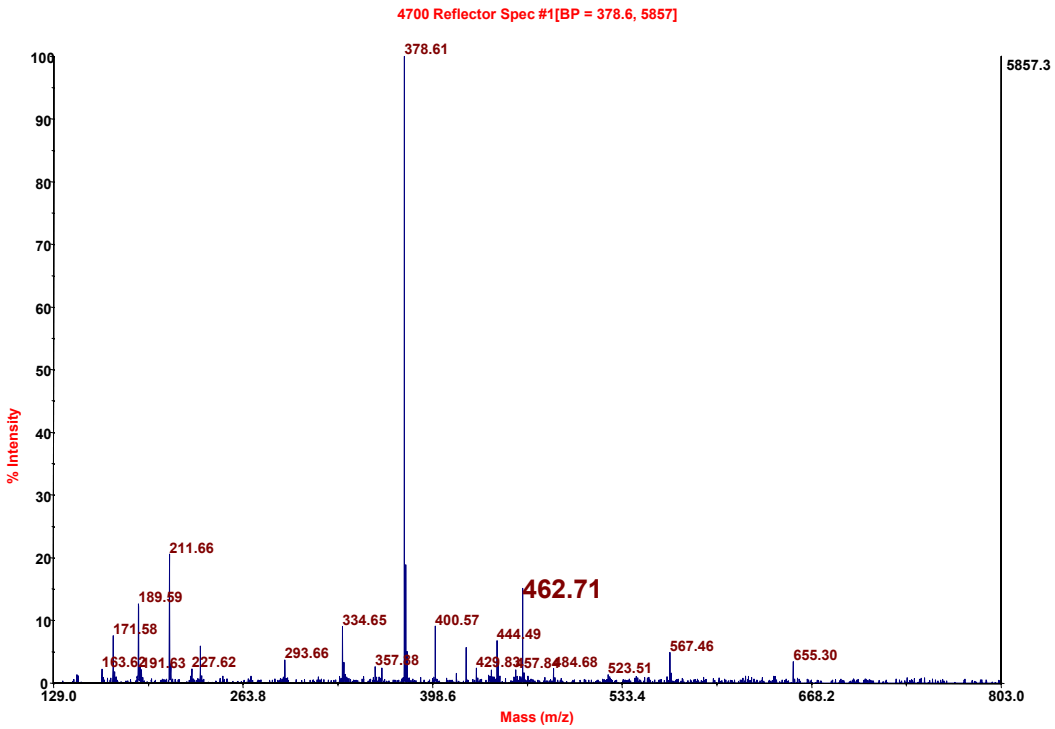
B Supplemental material Chapter 4

Table A.3. Mass confirmations of peptide oligomers from Chapter 4 via MALDI-ToF/MS

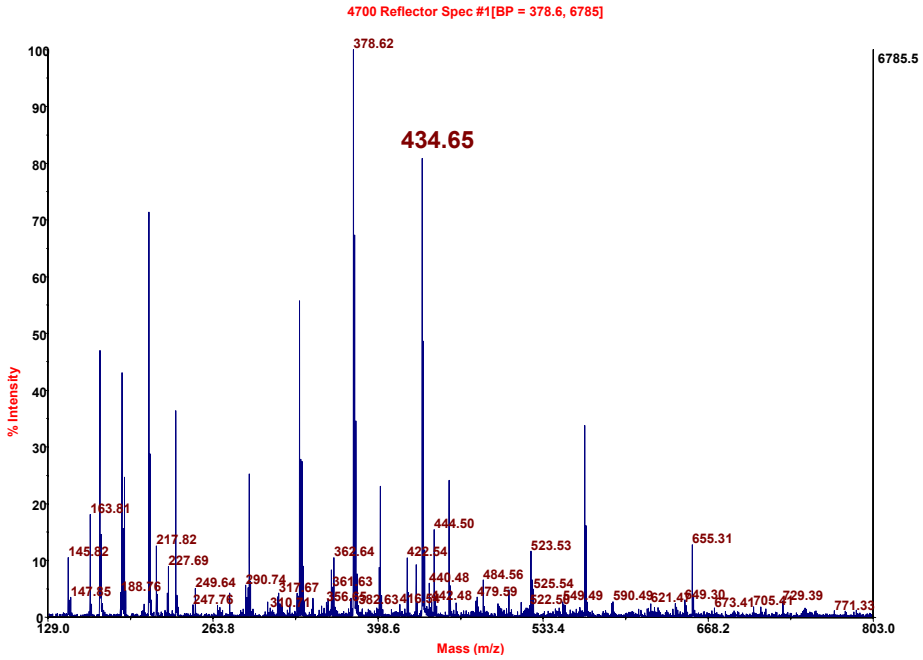
Bz-Arg



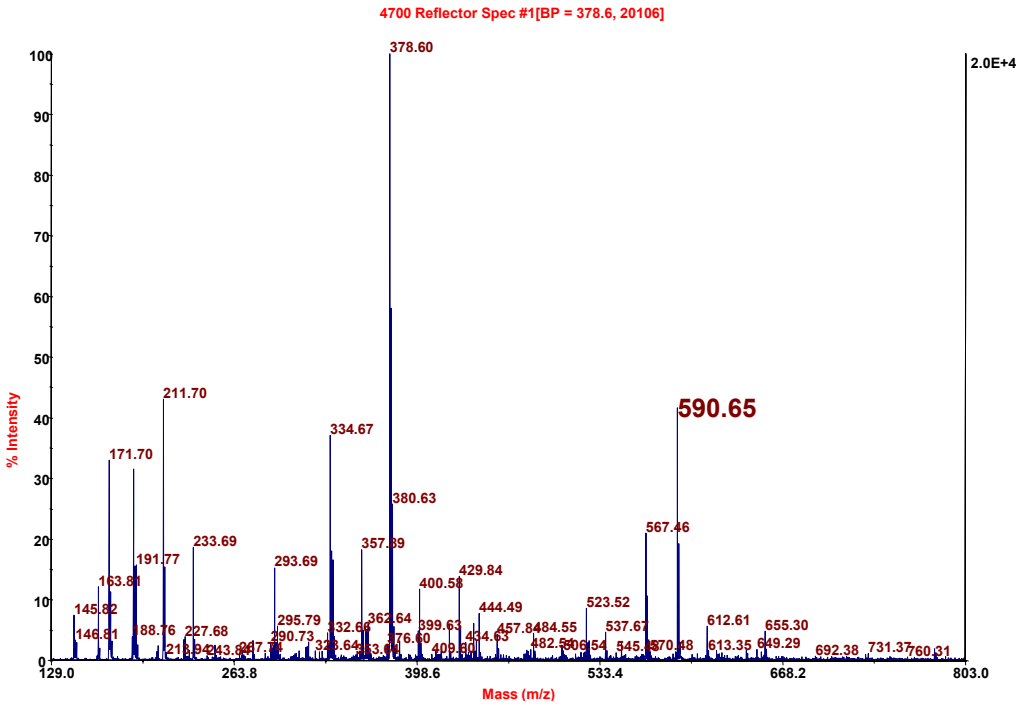
Bz-Arg-Arg-OEt



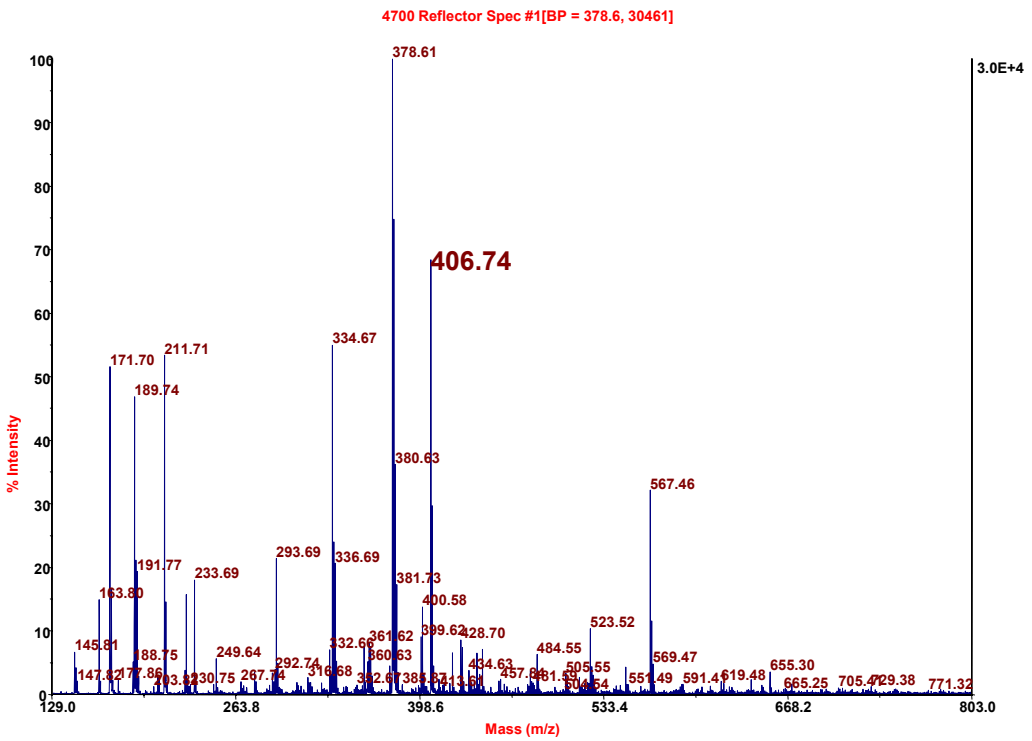
Bz-Arg-Arg



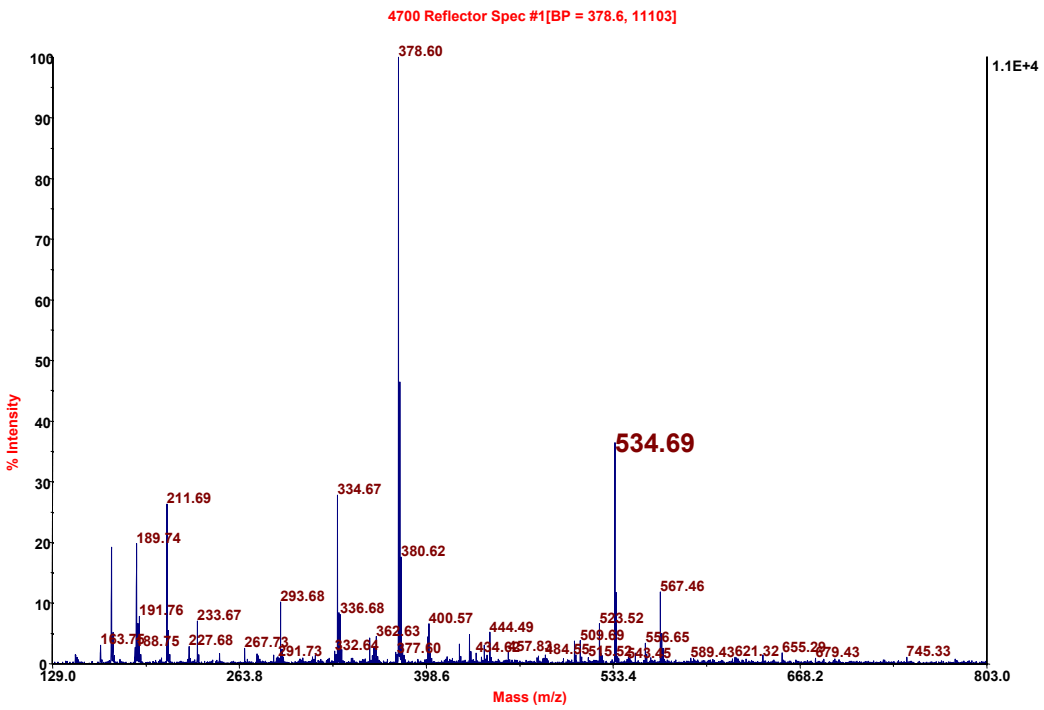
Bz-Arg-Arg-Arg



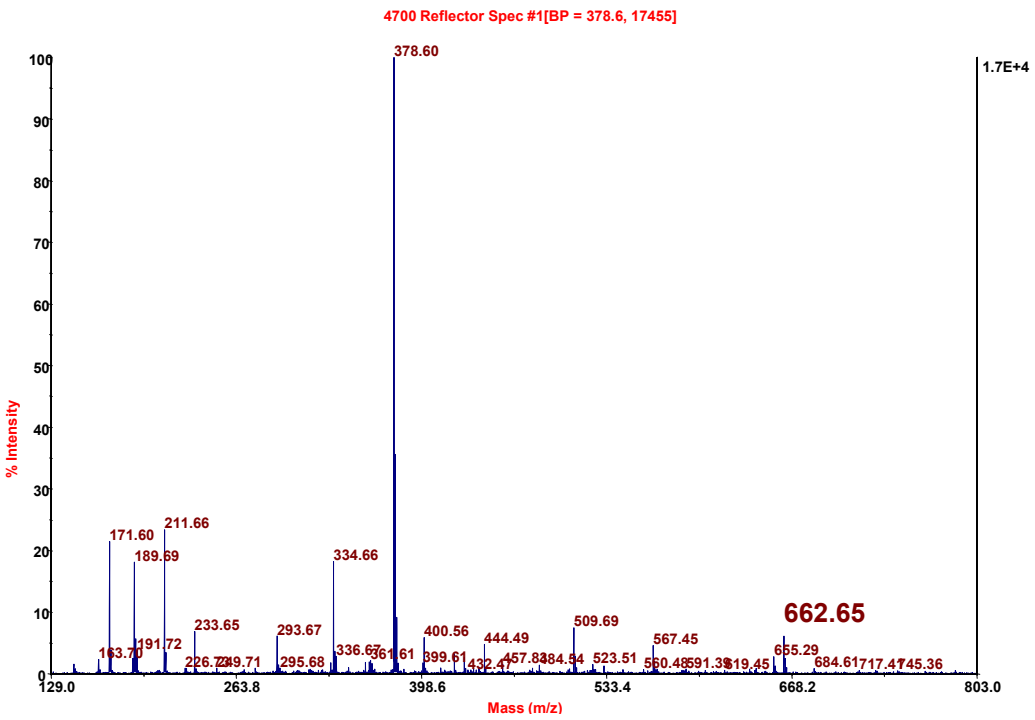
Bz-Arg-Lys



Bz-Arg-Lys-Lys



Bz-
Arg-
Lys-
Lys-
Lys



List of references

Abian, O., Mateo, C., Fernandez-Lorente, G., Palomo, J. M., Fernandez-Lafuente, R. and Guisan, J. M. (2001). "Stabilization of immobilized enzymes against water-soluble organic cosolvents and generation of hyper-hydrophilic micro-environments surrounding enzyme molecules." Biocatalysis and Biotransformation 19(5-6): 489-503.

Ahmad, I., Perkins, W. R., Lupan, D. M., Selsted, M. E. and Janoff, A. S. (1995). "Liposomal entrapment of the neutrophil-derived peptide indolicidin endows it with in vivo antifungal activity." Biochimica et Biophysica Acta (BBA) - Biomembranes 1237(2): 109-114.

Andre, M., Kühl, B., Brenner-Weiss, G., Syldatk, C. and Rudat, J. (2014). "Cationic heterooligopeptides by ficain-catalyzed co-oligomerization of lysine and methionine ethylesters." Journal of Peptide Science 20(8): 625-629.

Arica, M. Y., Yavuz, H. and Denizli, A. (2001). "Immobilization of glucoamylase on the plain and on the spacer arm-attached poly(HEMA-EGDMA) microspheres." Journal of Applied Polymer Science 81(11): 2702-2710.

Arica, M. Y., Altintas, B. and Bayramoglu, G. (2009). "Immobilization of laccase onto spacer-arm attached non-porous poly(GMA/EGDMA) beads: Application for textile dye degradation." Bioresource Technology 100(2): 665-669.

Atkinson, B. and Davies, I. J. (1974). "Overall rate of substrate uptake (reaction) by microbial Films.1. Biological rate equation." Transactions of the Institution of Chemical Engineers 52(3): 248-259.

Axelsson, I. (1978). "Characterization of proteins and other macromolecules by agarose gel chromatography." Journal of Chromatography A 152(1): 21-32.

Ayhan, F., Ayhan, H., Piskin, E. and Tanyolac, A. (2002). "Optimization of urease immobilization onto non-porous HEMA incorporated poly(EGDMA) microbeads and estimation of kinetic parameters." Bioresource Technology 81(2): 131-140.

Barros, R. J., Wehtje, E., Garcia, F. A. P. and Adlercreutz, P. (1998). "Physical characterization of porous materials and correlation with the activity of immobilized enzyme in organic medium." Biocatalysis and Biotransformation 16(1): 67-85.

Benns, J. M., Choi, J. S., Mahato, R. I., Park, J. S. and Kim, S. W. (2000). "pH-sensitive cationic polymer gene delivery vehicle: N-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer." Bioconjugate Chemistry 11(5): 637-645.

Berg, J. M. and Tymoczko, J. L. (2012). Stryer Biochemie. Stanford, Spektrum.

Betancor, L., Fuentes, M., Dellamora-Ortiz, G., Lopez-Gallego, F., Hidalgo, A., Alonso-Morales, N., Mateo, C., Guisan, J. M. and Fernandez-Lafuente, R. (2005). "Dextran aldehyde coating of glucose oxidase immobilized on magnetic nanoparticles prevents its inactivation by gas bubbles." Journal of Molecular Catalysis B: Enzymatic 32(3): 97-101.

Bongers, J. and Heimer, E. P. (1994). "Recent applications of enzymatic peptide-synthesis." Peptides 15(1): 183-193.

Bortone, N., Fidaleo, M. and Moresi, M. (2012). "Immobilization/stabilization of acid urease on Eupergit® supports." Biotechnology Progress 28(5): 1232-1244.

Bortone, N., Fidaleo, M. and Moresi, M. (2013). "Assessment of diffusion limitations on the performance of immobilised acid urease derivatives." Chemical Engineering 32: 1129-1134.

Bozhinova, D., Galunsky, B., Yueping, G., Franzreb, M., Koster, R. and Kasche, V. (2004). "Evaluation of magnetic polymer micro-beads as carriers of immobilised biocatalysts for selective and stereoselective transformations." Biotechnology Letters 26(4): 343-350.

Buchholz, K., Kasche, V. and Bornscheuer, U. T. (2005). Biocatalysts and enzyme technology. Weinheim, Wiley-VCH.

Bulmus, V., Ayhan, H. and Piskin, E. (1997). "Modified PMMA monosize microbeads for glucose oxidase immobilization." Chemical Engineering Journal 65(1): 71-76.

Cao, L. Q., Van Langen, L. and Sheldon, R. A. (2003). "Immobilised enzymes: carrier-bound or carrier-free?" Current Opinion in Biotechnology 14(4): 387-394.

Cao, L. Q. (2005). "Immobilised enzymes: science or art?" Current Opinion in Chemical Biology 9(2): 217-226.

Cavalcante, A. H. M., Carvalho, L. B. and Carneiro-Da-Cunha, M. G. (2006). "Cellulosic exopolysaccharide produced by *Zoogloea sp.* as a film support for trypsin immobilisation." Biochemical Engineering Journal 29(3): 258-261.

Chaplin, M. F. and Bucke, C. (1990). Enzyme technology. Cambridge, Cambridge University Press.

Chen, B., Dingerdissen, U., Krauter, J. G. E., Lansink Rotgerink, H. G. J., Möbus, K., Ostgard, D. J., Panster, P., Riermeier, T. H., Seebald, S., Tacke, T. and Trauthwein, H. (2005). "New developments in hydrogenation catalysis particularly in synthesis of fine and intermediate chemicals." Applied Catalysis A: General 280(1): 17-46.

Chen, B., Hu, J., Miller, E. M., Xie, W. C., Cai, M. M. and Gross, R. A. (2008). "*Candida antarctica* lipase B chemically immobilized on epoxy-activated micro- and nanobeads: catalysts for polyester synthesis." Biomacromolecules 9(2): 463-471.

Chen, J.-P. and Su, D.-R. (2001). "Latex particles with thermo-flocculation and magnetic properties for immobilization of α-chymotrypsin." Biotechnology Progress 17(2): 369-375.

Chen, J. P. and Lin, W. S. (2003). "Sol-gel powders and supported sol-gel polymers for immobilization of lipase in ester synthesis." Enzyme and Microbial Technology 32(7): 801-811.

Clark, D. S. and Bailey, J. E. (2002). "Structure-function relationships in immobilized chymotrypsin catalysis." Biotechnology and Bioengineering 79(5): 539-549.

Costantino, H. R., Griebenow, K., Langer, R. and Klibanov, A. M. (1997). "On the pH memory of lyophilized compounds containing protein functional groups." Biotechnology and Bioengineering 53(3): 345-348.

Damköhler, G. (1937). "Einflüsse der Strömung, Diffusion und des Wärmeüberganges auf die Leistung von Reaktionsöfen: II. Die isotherme, raumbeständige, homogene Reaktion erster Ordnung." Zeitschrift für Elektrochemie und angewandte physikalische Chemie 43(1): 1-8.

De Castro, H. F., De Oliveira, P. C., Soares, C. M. F. and Zanin, G. M. (1999). "Immobilization of porcine pancreatic lipase on celite for application in the synthesis of butyl butyrate in a nonaqueous system." Journal of the American Oil Chemists Society 76(1): 147-152.

De Maio, A., El-Masry, M., Portaccio, M., Diano, N., Di Martino, S., Mattei, A., Bencivenga, U. and Mita, D. (2003). "Influence of the spacer length on the activity of enzymes immobilised on nylon/polyGMA membranes: part 1. Isothermal conditions." Journal of Molecular Catalysis B: Enzymatic 21(4): 239-252.

Dejaegher, B. and Vander Heyden, Y. (2011). "Experimental designs and their recent advances in set-up, data interpretation, and analytical applications." Journal of Pharmaceutical and Biomedical Analysis 56(2): 141-158.

Deming, T. J. (2007). "Synthetic polypeptides for biomedical applications." Progress in Polymer Science 32(8-9): 858-875.

Dicosimo, R., Mcauliffe, J., Poulose, A. J. and Bohlmann, G. (2013). "Industrial use of immobilized enzymes." Chemical Society Reviews 42(15): 6437-6474.

Dincer, A. and Telefoncu, A. (2007). "Improving the stability of cellulase by immobilization on modified polyvinyl alcohol coated chitosan beads." Journal of Molecular Catalysis B-Enzymatic 45(1-2): 10-14.

Du, T. X., Liu, B. L., Hou, X. H., Zhang, B. T. and Du, C. M. (2009). "Covalent immobilization of glucose oxidase onto Poly(St-GMA-NaSS) monodisperse microspheres via BSA as spacer arm." Applied Surface Science 255(18): 7937-7941.

Emneus, J. and Gorton, L. (1993). "Comparison between different inorganic supports for the immobilization of amyloglucosidase and α-amylase to be used in enzyme reactors in flow-injection systems: Part II. Hydrolysis of glycogen." Analytica Chimica Acta 276(2): 319-328.

Engasser, J.-M. and Horvath, C. (1973). "Effect of internal diffusion in heterogeneous enzyme systems: Evaluation of true kinetic parameters and substrate diffusivity." Journal of Theoretical Biology 42(1): 137-155.

Fairman, R. and Akerfeldt, K. S. (2005). "Peptides as novel smart materials." Current Opinion in Structural Biology 15(4): 453-463.

Frey, P. A. and Hegeman, A. D. (2007). Enzymatic reaction mechanisms. New York, Oxford University Press.

Furukawa, S.-Y., Ono, T., Ijima, H. and Kawakami, K. (2002). "Effect of imprinting sol-gel immobilized lipase with chiral template substrates in esterification of (R)-(+)- and (S)-(-)-glycidol." Journal of Molecular Catalysis B: Enzymatic 17(1): 23-28.

Gao, S. L., Wang, Y. J., Wang, T., Luo, G. S. and Dai, Y. Y. (2009). "Immobilization of lipase on methyl-modified silica aerogels by physical adsorption." Bioresource Technology 100(2): 996-999.

Garcia-Galan, C., Berenguer-Murcia, A., Fernandez-Lafuente, R. and Rodrigues, R. C. (2011). "Potential of different enzyme immobilization strategies to improve enzyme performance." Advanced Synthesis & Catalysis 353(16): 2885-2904.

Gast, F. U., Franke, I., Meiss, G. and Pingoud, A. (2001). "Immobilization of sugar-non-specific nucleases by utilizing the streptavidin-biotin interaction." Journal of Biotechnology 87(2): 131-141.

Giessen, T. W. and Marahiel, M. A. (2012). "Ribosome-independent biosynthesis of biologically active peptides: Application of synthetic biology to generate structural diversity." Febs Letters 586(15): 2065-2075.

Gill, I., Lopezfandino, R., Jorba, X. and Vulfson, E. N. (1996). "Biologically active peptides and enzymatic approaches to their production." Enzyme and Microbial Technology 18(3): 162-183.

Gleeson, O., Davies, G.-L., Peschiulli, A., Tekoriute, R., Gun'ko, Y. K. and Connon, S. J. (2011). "The immobilisation of chiral organocatalysts on magnetic nanoparticles: the support particle cannot always be considered inert." Organic & Biomolecular Chemistry 9(22): 7929-7940.

Goddard, J. M. and Hotchkiss, J. H. (2007). "Polymer surface modification for the attachment of bioactive compounds." Progress in Polymer Science 32(7): 698-725.

Gruber, H. J., Marek, M., Schindler, H. and Kaiser, K. (1997). "Biotin-fluorophore conjugates with poly(ethylene glycol) spacers retain intense fluorescence after binding to avidin and streptavidin." Bioconjugate Chemistry 8(4): 552-559.

Guzman, F., Barberis, S. and Illanes, A. (2007). "Peptide synthesis: chemical or enzymatic." Electronic Journal of Biotechnology 10(2): 279-314.

Halling, P. J. and Dunnill, P. (1980). "Magnetic supports for immobilized enzymes and bioaffinity adsorbents." Enzyme and Microbial Technology 2(1): 2-10.

Hanisch, U.-K., Könnecke, A., Schellenberger, V. and Jakubke, H.-D. (1987). "Characterization of the S'subsite specificity of trypsin." Biocatalysis and Biotransformation 1(2): 129-136.

He, F., Wang, Y. X., Feng, J., Zhuo, R. X. and Wang, X. L. (2003). "Synthesis of poly [(5-benzyloxy-trimethylene carbonate)-co-(5,5 dimethyl-trimethylene carbonate)] catalyzed by immobilized lipase on silica particles with different size." Polymer 44(11): 3215-3219.

Hermanson, G. (2008). Bioconjugate techniques. London, Academic Press Elsevier Inc.

Herndl, G. and Mersmann, A. B. (1981). "Fluid-dynamics and mass-transfer in stirred suspensions." Chemical Engineering Communications 13(1-3): 23-37.

Hinterwirth, H., Lindner, W. and Lammerhofer, M. (2012). "Bioconjugation of trypsin onto gold nanoparticles: Effect of surface chemistry on bioactivity." Analytica Chimica Acta 733: 90-97.

Hoffmann, C. (2003). Einsatz magnetischer Separationsverfahren zur biotechnologischen Produktaufarbeitung. Forschungszentrum Karlsruhe in der Helmholtz-Gemeinschaft, FZKA ; 6915. Karlsruhe, Forschungszentrum Karlsruhe GmbH: VII, 188 S.

Illanes, A. (2008). Enzyme biocatalysis. Chile, Springer.

Itoyama, K., Tanibe, H., Hayashi, T. and Ikada, Y. (1994). "Spacer effects on enzymatic-activity of papain immobilized onto porous chitosan beads." Biomaterials 15(2): 107-112.

Jia, H., Zhu, G. and Wang, P. (2003). "Catalytic behaviors of enzymes attached to nanoparticles: the effect of particle mobility." Biotechnology and Bioengineering 84(4): 406-414.

Johnson, K. D., Clark, A. and Marshall, S. (2002). "A functional comparison of ovine and porcine trypsins." Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 131(3): 423-431.

Kalantari, M., Kazemeini, M., Tabandeh, F. and Arpanaei, A. (2012). "Lipase immobilisation on magnetic silica nanocomposite particles: effects of the silica structure on properties of the immobilised enzyme." Journal of Materials Chemistry 22(17): 8385-8393.

Kasche, V. (1983). "Correlation of experimental and theoretical data for artificial and natural systems with immobilized biocatalysts." Enzyme and Microbial Technology 5(1): 2-13.

Kastin, A. (2013). Handbook of biologically active peptides. New York, Academic Press.

Kheirolomoom, A., Khorasheh, F. and Fazelinia, H. (2002). "Influence of external mass transfer limitation on apparent kinetic parameters of penicillin G acylase immobilized on nonporous ultrafine silica particles." Journal of Bioscience and Bioengineering 93(2): 125-129.

Kim, J., Grate, J. W. and Wang, P. (2006). "Nanostructures for enzyme stabilization." Chemical Engineering Science 61(3): 1017-1026.

Klibanov, A. M. (2001). "Improving enzymes by using them in organic solvents." Nature 409(6817): 241-246.

Koneracka, M., Kopcansky, P., Timko, M., Ramchand, C. N., Saiyed, Z. M., Trevan, M. and De Sequeira, A. (2006). Immobilization of enzymes on magnetic particles. Immobilization of enzymes and cells. J. M. Guisan. Totowa, Human Press Inc.: 217-228.

Kraut, J. (1977). "Serine proteases: structure and mechanism of catalysis." Annual review of biochemistry 46(1): 331-358.

Li, D. F., Ding, H. C. and Zhou, T. (2013). "Covalent immobilization of mixed proteases, trypsin and chymotrypsin, onto modified polyvinyl chloride microspheres." Journal of Agricultural and Food Chemistry 61(44): 10447-10453.

Li, G., Raman, V. K., Xie, W. C. and Gross, R. A. (2008). "Protease-catalyzed co-oligomerizations of L-leucine ethyl ester with L-glutamic acid diethyl ester: Sequence and chain length distributions." Macromolecules 41(19): 7003-7012.

Liao, H. D., Yuan, L., Tong, C. Y., Zhu, Y. H., Li, D. and Liu, X. M. (2008). "Immobilization of cellulase based on polyvinyl alcohol/Fe_2O_3 nanoparticles." Chemical Journal of Chinese Universities-Chinese 29(8): 1564-1568.

Liao, H. D., Chen, D., Yuan, L., Zheng, M., Zhu, Y. H. and Liu, X. M. (2010). "Immobilized cellulase by polyvinyl alcohol/Fe_2O_3 magnetic nanoparticle to degrade microcrystalline cellulose." Carbohydrate Polymers 82(3): 600-604.

Liese, A., Seelbach, K. and Wandrey, C. (2006). Industrial biotransformations. Weinheim, Wiley-VCH.

Lu, A.-H., Salabas, E. L. and Schüth, F. (2007). "Magnetic nanoparticles: synthesis, protection, functionalization and application." Angewandte Chemie International Edition 46(8): 1222-1244.

Lupo, M. P. and Cole, A. L. (2007). "Cosmeceutical peptides." Dermatologic Therapy 20(5): 343-349.

Macario, A. and Giordano, G. (2013). "Catalytic conversion of renewable sources for biodiesel production: a comparison between biocatalysts and inorganic catalysts." Catalysis Letters 143(2): 159-168.

Magario, I., Ma, X., Neumann, A., Syldatk, C. and Hausmann, R. (2008). "Non-porous magnetic micro-particles: Comparison to porous enzyme carriers for a diffusion rate-controlled enzymatic conversion." Journal of Biotechnology 134(1-2): 72-78.

Malmsten, M. and Larsson, A. (2000). "Immobilization of trypsin on porous glycidyl methacrylate beads: effects of polymer hydrophilization." Colloids and Surfaces B-Biointerfaces 18(3-4): 277-284.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M. and Fernandez-Lafuente, R. (2007). "Improvement of enzyme activity, stability and selectivity via immobilization techniques." Enzyme and Microbial Technology 40(6): 1451-1463.

Montes, T., Grazú, V., Manso, I., Galán, B., López-Gallego, F., González, R., Hermoso, J. A., García, J. L., Guisán, J. M. and Fernández-Lafuente, R. (2007). "Improved stabilization of genetically modified Penicillin G acylase in the presence of organic cosolvents by co-immobilization of the enzyme with polyethyleneimine." Advanced Synthesis & Catalysis 349(3): 459-464.

Morihara, K., Oka, T. and Tsuzuki, H. (1983). "Semi-synthesis of human insulin."Shionogi & Co., Ltd. 4320196.

Mukherjee, A. K., Kumar, T. S., Rai, S. K. and Roy, J. K. (2010). "Statistical optimization of *Bacillus alcalophilus* alpha-amylase immobilization on iron-oxide magnetic nanoparticles." Biotechnology and Bioprocess Engineering 15(6): 984-992.

Muller, R., Glaser, S., Geipel, F., Thalhofer, J. P., Rexer, B., Schneider, C., Ratka, M., Ronning, S., Eckstein, H. and Giessel, C. (2007). "Method for producing recombinant trypsin."Roche Diagnostics Operations, Inc. 7276605.

Myers, M., Connor, E. F., Glauser, T., Mock, A., Nyce, G. and Hedrick, J. L. (2002). "Phosphines: nucleophilic organic catalysts for the controlled ring-opening polymerization of lactides." Journal of Polymer Science Part a-Polymer Chemistry 40(7): 844-851.

Netto, C. G. C. M., Toma, H. E. and Andrade, L. H. (2012). "Superparamagnetic nanoparticles as versatile carriers and supporting materials for enzymes." Journal of Molecular Catalysis B: Enzymatic 85-86: 71-92.

Nie, G., Chen, Z., Zheng, Z., Jin, W., Gong, G., Wang, L. and Yue, W. (2013). "Enhancement of transesterification-catalyzing capability of bio-imprinted tannase in organic solvents by cryogenic protection and immobilization." Journal of Molecular Catalysis B: Enzymatic 94(0): 1-6.

Nouaimi, M., Möschel, K. and Bisswanger, H. (2001). "Immobilization of trypsin on polyester fleece via different spacers." Enzyme and Microbial Technology 29(8-9): 567-574.

Oh, J.-T. and Kim, J.-H. (2000). "Preparation and properties of immobilized amyloglucosidase on nonporous PS/PNaSS microspheres." Enzyme and Microbial Technology 27(6): 356-361.

Opstal, T., Couchez, K. and Verpoort, F. (2003). "Easily accessible ring opening metathesis and atom transfer radical polymerization catalysts based on arene, norbornadiene and cyclooctadiene ruthenium complexes bearing Schiff base ligands." Advanced Synthesis & Catalysis 345(3): 393-401.

Ozturk, T. K. and Kilinc, A. (2010). "Immobilization of lipase in organic solvent in the presence of fatty acid additives." Journal of Molecular Catalysis B: Enzymatic 67(3-4): 214-218.

Ozyilmaz, G. (2009). "The effect of spacer arm on hydrolytic and synthetic activity of *Candida rugosa* lipase immobilized on silica gel." Journal of Molecular Catalysis B-Enzymatic 56(4): 231-236.

Peißker, F. and Fischer, L. (1999). "Crosslinking of imprinted proteases to maintain a tailor-made substrate selectivity in aqueous solutions." Bioorganic & medicinal chemistry 7(10): 2231-2237.

Pencreach, G., Leullier, M. and Baratti, J. C. (1997). "Properties of free and immobilized lipase from *Pseudomonas cepacia*." Biotechnology and Bioengineering 56(2): 181-189.

Penzol, G., Armisen, P., Fernandez-Lafuente, R., Rodes, L. and Guisan, J. M. (1998). "Use of dextrans as long and hydrophilic spacer arms to improve the performance of immobilized proteins acting on macromolecules." Biotechnology and Bioengineering 60(4): 518-523.

Pich, A., Bhattacharya, S., Adler, H.-J. P., Wage, T., Taubenberger, A., Li, Z., Van Pee, K.-H., Böhmer, U. and Bley, T. (2006). "Composite magnetic particles as

carriers for laccase from *Trametes versicolor*." Macromolecular Bioscience 6(4): 301-310.
Pugniere, M., Skalli, A., Coletti-Previero, M.-A. and Previero, A. (1986). "Peptide and ester synthesis in organic solvents catalyzed by seryl proteases linked to alumina." Proteins: Structure, Function, and Bioinformatics 1(2): 134-138.
Qin, X., Xie, W. C., Su, Q., Du, W. Z. and Gross, R. A. (2011). "Protease-catalyzed oligomerization of L-lysine ethyl ester in aqueous solution." ACS Catalysis 1(9): 1022-1034.
Qin, X., Khuong, A. C., Yu, Z., Du, W. Z., Decatur, J. and Gross, R. A. (2013). "Simplifying alternating peptide synthesis by protease-catalyzed dipeptide oligomerization." Chemical Communications 49(4): 385-387.
Quaedflieg, P. J. L. M., Sonke, T. and Wagner, A. F. V. (2003). "Synthesis and recovery of aspartame involving enzymatic deformylation step."Holland Sweetener Company, V.O.F. 6617127.
Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). "Molecular and biotechnological aspects of microbial proteases." Microbiology and Molecular Biology Reviews 62(3): 597-635.
Rawlings, N. D., Morton, F. R. and Barrett, A. J. (2007). An introduction to peptidases and the Merops database. Industrial enzymes. J. Polaina and A. P. Maccabe, Springer Netherlands.
Reichardt, C. (2004). Empirical parameters of solvent polarity. Solvents and solvent effects in organic chemistry, Wiley-VCH Verlag GmbH & Co. KGaA: 389-469.
Resindion (2015). "ReliZyme™ EA403 technical data sheet." Retrieved 06/2015, from http://www.resindion.com/.
Robinson, P. J., Dunnill, P. and Lilly, M. D. (1973). "The properties of magnetic supports in relation to immobilized enzyme reactors." Biotechnology and Bioengineering 15(3): 603-606.
Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, A., Torres, R. and Fernandez-Lafuente, R. (2012). "Modifying enzyme activity and selectivity by immobilization." Chemical Society Reviews 42(15): 6290-6307.
Russell, A. J., Chatterjee, S., Rapanovich, I. and Goodwin, J., J. G. (1992). Mechanistic enzymology in anhydrous organic solvents. Biomolecules in organic solvents. A. Gomez-Puyou. Boca Raton, Florida, USA, CRC press: 92-94.
Schechter, I. and Berger, A. (1967). "On size of active site in proteases. I. Papain." Biochemical and Biophysical Research Communications 27(2): 157-162.
Schellenberger, V. and Jakubke, H.-D. (1991). "Protease-catalyzed kinetically controlled peptide synthesis." Angewandte Chemie International Edition in English 30(11): 1437-1449.
Sears, P. S. and Clark, D. S. (1993). "Comparison of soluble and immobilized trypsin kinetics - implications for peptide-synthesis." Biotechnology and Bioengineering 42(1): 118-124.
Sergeeva, M. V., Paradkar, V. M. and Dordick, J. S. (1997). "Peptide synthesis using proteases dissolved in organic solvents." Enzyme and Microbial Technology 20(8): 623-628.
Sharon, J. L. and Puleo, D. A. (2008). "The use of N-terminal immobilization of PTH(1-34) on PLGA to enhance bioactivity." Biomaterials 29(21): 3137-3142.
Sheldon, R. A. (2007). "Enzyme immobilization: the quest for optimum performance." Advanced Synthesis & Catalysis 349(8-9): 1289-1307.
Siso, M. I. G., Graber, M., Condoret, J.-S. and Combes, D. (1990). "Effect of diffusional resistances on the action pattern of immobilized alpha-amylase." Journal of Chemical Technology & Biotechnology 48(2): 185-200.
Soares, C. M. F., De Castro, H. F., De Moraes, F. F. and Zanin, G. M. (1999). "Characterization and utilization of *Candida rugosa* lipase immobilized on controlled pore silica." Applied Biochemistry and Biotechnology 77-9: 745-757.
Stark, M.-B. and Holmberg, K. (1989). "Covalent immobilization of lipase in organic solvents." Biotechnology and Bioengineering 34(7): 942-950.
Sun, J. N., Jiang, Y. J., Zhou, L. Y. and Gao, J. (2010). "Immobilization of *Candida antarctica* lipase B by adsorption in organic medium." New Biotechnology 27(1): 53-58.

Thiele, E. W. (1939). "Relation between catalytic activity and size of particle." Industrial & Engineering Chemistry 31(7): 916-920.
Tischer, W. and Kasche, V. (1999). "Immobilized enzymes: crystals or carriers?" Trends in Biotechnology 17(8): 326-335.
Truskey, G. A., Yuan, F. and Katz, D. F. (2004). Transport phenomena in biological systems, Pearson/Prentice Hall.
Tufvesson, P., Lima-Ramos, J., Nordblad, M. and Woodley, J. M. (2011). "Guidelines and cost analysis for catalyst production in biocatalytic processes." Organic Process Research & Development 15(1): 266-274.
Vertegel, A. A., Siegel, R. W. and Dordick, J. S. (2004). "Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme." Langmuir 20(16): 6800-6807.
Viswanathan, K., Omorebokhae, R., Li, G. and Gross, R. A. (2010). "Protease-catalyzed oligomerization of hydrophobic amino acid ethyl esters in homogeneous reaction media using L-phenylalanine as a model system." Biomacromolecules 11(8): 2152-2160.
Walas, S. (1997). Chemical reactors. Perry's Chemical Engineers' Handbook, Seventh Edition. D. Green and R. Perry. New York, McGraw-Hill Education: 23-29.
Weisz, P. and Hicks, J. (1962). "The behaviour of porous catalyst particles in view of internal mass and heat diffusion effects." Chemical Engineering Science 17(4): 265-275.
Weisz, P. B. (1973). "Diffusion and chemical transformation." Science 179(4072): 433-440.
Wheeler, A. (1951). "Reaction rates and selectivity in catalyst pores." Advances in Catalysis 3: 249-327.
Woldike, H. F. and Kjeldsen, T. B. (1999). "Process for producing trypsin (trypsinogen)."Novo Nordisk A/S (Bagsvaerd, DK)
Wu, Y., Wang, Y., Luo, G. and Dai, Y. (2009). "In situ preparation of magnetic Fe_3O_4-chitosan nanoparticles for lipase immobilization by cross-linking and oxidation in aqueous solution." Bioresource Technology 100(14): 3459-3464.
Yamamoto, S., Imamura, A., Susanti, I., Hori, K., Tanji, Y. and Unno, H. (2005). "Effect of spacer length on beta-lactoglobulin hydrolysis by trypsin covalently immobilized on a cellulosic support." Food and Bioproducts Processing 83(C1): 61-67.
Yamane, T. (1981). "On approximate expressions of effectiveness factors for immobilized biocatalysts." Journal of Fermentation Technology 59(5): 375-381.
Yang, Z., Zacherl, D. and Russell, A. J. (1993). "pH-dependence of subtilisin dispersed in organic-solvents." Journal of the American Chemical Society 115(26): 12251-12257.
Yee, L. and Blanch, H. (1993). "Recombinant trypsin production in high cell density fed-batch cultures in *Escherichia coli*." Biotechnology and Bioengineering 41(8): 781-790.
Zaks, A. and Klibanov, A. M. (1988). "Enzymatic catalysis in nonaqueous solvents." Journal of Biological Chemistry 263(7): 3194-3201.
Zhang, D. H., Li, Y. Q., Peng, L. J. and Chen, N. (2014). "Lipase immobilization on magnetic microspheres via spacer arms: Effect of steric hindrance on the activity." Biotechnology and Bioprocess Engineering 19(5): 838-843.
Zhang, Y. D., Zhang, Y., Jiang, J. J., Li, L., Yu, C. H. and Hei, T. T. (2011). "Surface derivatization with spacer molecules on glutaraldehyde-activated amino-microplates for covalent immobilization of beta-glucosidase." Applied Surface Science 257(7): 2712-2716.
Zhou, C., Wu, X. M., Jiang, B. and Shen, S. B. (2011). "Immobilization strategy of accessible transmission for trypsin to catalyze synthesis of dipeptide in mesoporous support." Korean Journal of Chemical Engineering 28(12): 2300-2305.
Zhu, X., Zhou, T., Wu, X., Cai, Y., Yao, D., Xie, C. and Liu, D. (2011). "Covalent immobilization of enzymes within micro-aqueous organic media." Journal of Molecular Catalysis B: Enzymatic 72(3-4): 145-149.

Zhu, Y., Kaskel, S., Shi, J., Wage, T. and Van Pee, K.-H. (2007). "Immobilization of *Trametes versicolor* laccase on magnetically separable mesoporous silica spheres." Chemistry of Materials 19(26): 6408-6413.