

## Reihe 5

Grund- und  
Werkstoffe/  
Kunststoffe

Nr. 760

Dipl.-Chem. Ludmilla Derr,  
Bremen

## Interactions between enzymes and oxide colloidal particles and their influence on enzymatic activity



# **Interactions between enzymes and oxide colloidal particles and their influence on enzymatic activity**

Dem Fachbereich Produktionstechnik

der

**UNIVERSITÄT BREMEN**

zur Erlangung des Grades  
Doktor-Ingenieur  
vorgelegte

Dissertation

von

Diplom-Chemikerin

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Tag der mündlichen Prüfung: 11. März 2016



# Fortschritt-Berichte VDI

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VDI verlag

Derr, Ludmilla

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Fortschr.-Ber. VDI Reihe 5 Nr. 760. Düsseldorf: VDI Verlag 2016.

210 Seiten, 66 Bilder, 9 Tabellen.

ISBN 978-3-18-376005-3, ISSN 0178-952X,

€ 76,00/VDI-Mitgliederpreis € 68,40.

**Keywords:**  $\alpha$ -chymotrypsin – Adsorption – Colloidal particles – Physisorption – Covalent immobilization – Enzyme immobilization – Enzymatic activity – Alumina – Silica – Titania

Enzyme immobilization on inorganic oxide particles is a widely employed technique that permits the reuse of costly enzymes in catalytic processes. The aim of this study was to investigate the effects of adsorption on ceramic particles on the catalytic activities of enzymes. The adsorption of the proteolytic enzyme  $\alpha$ -chymotrypsin on silica, alumina, and titania was studied. The enzyme adsorption was specifically investigated by material characterization before and after adsorption, quantification of the adsorbed enzyme and detailed enzymatic activity measurements. Furthermore, the experimental results were interpreted based on complementary simulations. Covalent enzyme immobilization on amino-functionalized alumina and silica was also performed. Its effects on the enzymatic activity of  $\alpha$ -chymotrypsin were additionally investigated by employing matrix-assisted laser desorption ionization time-of-flight mass spectroscopy.

### **Bibliographische Information der Deutschen Bibliothek**

Die Deutsche Bibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliographie; detaillierte bibliographische Daten sind im Internet unter <http://dnb.ddb.de> abrufbar.

### **Bibliographic information published by the Deutsche Bibliothek**

(German National Library)

The Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliographie (German National Bibliography); detailed bibliographic data is available via Internet at <http://dnb.ddb.de>.

Dissertation Universität Bremen

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Als Manuskript gedruckt. Printed in Germany.

ISSN 0178-952X

ISBN 978-3-18-376005-3

## Acknowledgments

I would like to acknowledge my thesis supervisor, Prof. Kurosch Rezwan, for the opportunity to complete this PhD thesis project and for the scientific freedom that he gave me during the years of my PhD studies.

I would also like to express my gratitude to my co-supervisor Prof. Jorg Thöming for being the co-examiner of this thesis.

Moreover, I would like to give special thanks to my collaborators Prof. Ralf Dringen, Prof. Lucio Colombi Ciacchi, Dr. Susan Köppen and Nils Hildebrand for motivating and valuable discussions.

Thanks to all colleagues from the working groups of Prof. Dringen and Prof. Colombi Ciacchi for their assistance, fruitful discussions and friendly collaboration over the course of numerous days in the laboratory.

Thanks to my officemates and friends Eduardo Meller, Karoline Pardun and Alieh Aminian for their support, for numerous scientific discussions and for a wonderfully friendly atmosphere in the office.

Furthermore, I would like to thank my colleagues Cristian Nuortila, Christian Ellenberg and Gabriela Berger for wonderful non-scientific discussions. Many thanks to all of my colleagues from the Advanced Ceramics Group for the friendly working atmosphere and their interest in my work.

I would like to thank my parents Tamara and Sergej Derr, my friends and my Kung Fu family for their support and their trust in me throughout the years of my PhD studies.

Last but certainly not least, I would like to thank Peter for his support, his motivation, his serenity, great discussions, and his constant trust in me over the last few years.

Ludmilla

*"Life can only be understood backwards; but it must be lived forwards."*

*Søren Kierkegaard*

*To M. F. C.*

# Contents

|   |       |
|---|-------|
| Acknowledgments   | III   |
| Contents  | V     |
| List of abbreviations and symbols   | XI    |
| Summary   | XVI   |
| Zusammenfassung   | XVIII |
| 1 Introduction  | 1     |
| 1.1 Aims and motivation   | 1     |
| 2 Interactions between colloidal particles in aqueous suspension                          | 5     |
| 2.1 Van der Waals forces  | 5     |
| 2.2 Electrostatic double layer forces   | 8     |
| 2.2.1 Surface charge  | 8     |
| 2.2.2 Zeta potential  | 11    |
| 2.2.3 The Poisson-Boltzmann equation  | 13    |
| 2.2.4 The electrostatic interaction energy between two spheres                            | 17    |
| 2.3 The DLVO theory   | 18    |
| 2.4 Non-DLVO forces   | 20    |
| 3 Proteins  | 22    |
| 3.1 The amino acid composition of proteins  | 22    |
| 3.2 Protein structure   | 23    |
| 3.3 Enzymes   | 24    |
| 3.3.1 $\alpha$ -Chymotrypsin  | 25    |
| 4 Colloidal particles, their surface functionalization and covalent enzyme immobilization | 27    |
| 4.1 Alumina colloidal particles   | 27    |
| 4.2 Silica colloidal particles  | 28    |

|       |  |    |
|-------|--|----|
| 4.3   | Titania colloidal particles  | 29 |
| 4.4   | Surface functionalization of colloidal particles   | 30 |
| 4.5   | Covalent enzyme immobilization   | 31 |
| 5     | Protein adsorption   | 34 |
| 5.1   | Influences on protein-particle interactions  | 34 |
| 5.2   | Thermodynamic approach   | 36 |
| 5.3   | Protein adsorption models  | 37 |
| 5.3.1 | The Langmuir adsorption model  | 37 |
| 5.3.2 | Other adsorption models  | 39 |
| 5.4   | Methods for studying protein-particle adsorption   | 39 |
| 6     | Enzymatic activity   | 41 |
| 6.1   | Michaelis-Menten kinetics  | 41 |
| 6.2   | Determination of $K_M$ and $V_{max}$ from linearization plots                                | 45 |
| 6.3   | Activity assays: principles  | 46 |
| 7     | Experimental methods and principles  | 48 |
| 7.1   | Electroacoustic spectroscopy: zeta potential   | 48 |
| 7.2   | Gas adsorption isotherms: specific surface area $SSA_{BET}$ , hydrophobicity/ hydrophilicity | 50 |
| 7.3   | UV-Vis spectroscopy: protein quantification  | 51 |
| 7.4   | Activity assessment of free and adsorbed $\alpha$ -chymotrypsin                              | 53 |
| 7.5   | Matrix-assisted laser desorption ionization time-of-flight mass spectroscopy                 | 55 |
| 7.6   | Circular dichroism spectroscopy: changes in protein structure                                | 56 |
| 7.7   | Visual molecular dynamics: modeling of proteins  | 58 |
| 8     | Physisorption of enzymatically active chymotrypsin on titania colloidal particles            | 59 |
| 8.1   | Introduction   | 59 |

|       |  |    |
|-------|--|----|
| 8.2   | Experimental section   | 61 |
| 8.2.1 | Materials  | 61 |
| 8.2.2 | Characterization of TiO <sub>2</sub> colloidal particles   | 61 |
| 8.2.3 | Hydrophobic/ hydrophilic properties of chymotrypsin  | 62 |
| 8.2.4 | Time-dependent and pH-dependent adsorption/ desorption of chymotrypsin   | 63 |
| 8.2.5 | Concentration-dependent adsorption of chymotrypsin on TiO <sub>2</sub>   | 65 |
| 8.2.6 | Determination of the enzymatic activity of chymotrypsin  | 65 |
| 8.2.7 | Statistical analysis   | 67 |
| 8.3   | Results  | 68 |
| 8.3.1 | Characterization of TiO <sub>2</sub> colloidal particles   | 68 |
| 8.3.2 | Time-dependent and pH-dependent adsorption/ desorption of chymotrypsin   | 70 |
| 8.3.3 | Concentration-dependent adsorption of chymotrypsin on TiO <sub>2</sub>   | 71 |
| 8.3.4 | Substrate concentration-dependency for free and adsorbed chymotrypsin  | 73 |
| 8.4   | Discussion   | 75 |
| 8.5   | Conclusions  | 79 |
| 8.6   | Acknowledgments  | 80 |
| 8.7   | Source   | 81 |
| 9     | Effects of silica and alumina colloidal particles on the enzymatic activity of $\alpha$ -chymotrypsin after adsorption | 82 |
| 9.1   | Introduction   | 82 |
| 9.2   | Experimental section   | 83 |
| 9.2.1 | Materials  | 83 |
| 9.2.2 | Characterization of SiO <sub>2</sub> and Al <sub>2</sub> O <sub>3</sub>  | 84 |
| 9.2.3 | Size determination and hydrophobic/ hydrophilic properties of chymotrypsin   | 85 |

|        |   |     |
|--------|---|-----|
| 9.2.4  | Adsorption and desorption studies of chymotrypsin   | 86  |
| 9.2.5  | Quantitative determination of adsorbed chymotrypsin   | 88  |
| 9.2.6  | Enzymatic activity measurements of free chymotrypsin  | 88  |
| 9.2.7  | Enzymatic activity measurements of adsorbed chymotrypsin  | 88  |
| 9.2.8  | Statistical analysis  | 89  |
| 9.3    | Results   | 90  |
| 9.3.1  | Characterization of SiO <sub>2</sub> and Al <sub>2</sub> O <sub>3</sub>   | 90  |
| 9.3.2  | Size determination and hydrophobic/ hydrophilic properties of chymotrypsin  | 92  |
| 9.3.3  | Adsorption of chymotrypsin on SiO <sub>2</sub> and Al <sub>2</sub> O <sub>3</sub> colloidal particles   | 93  |
| 9.3.4  | Adsorption/ desorption studies of chymotrypsin  | 94  |
| 9.3.5  | Enzymatic activity of adsorbed chymotrypsin   | 96  |
| 9.4    | Discussion  | 98  |
| 9.5    | Conclusions   | 101 |
| 9.6    | Acknowledgments   | 102 |
| 10     | Physisorption of $\alpha$ -chymotrypsin on SiO <sub>2</sub> and TiO <sub>2</sub> : a comparative study via experiments and molecular dynamics simulations | 103 |
| 10.1   | Introduction  | 103 |
| 10.2   | Materials and methods   | 106 |
| 10.2.1 | Materials   | 106 |
| 10.2.2 | Characterization of SiO <sub>2</sub> and TiO <sub>2</sub>   | 106 |
| 10.2.3 | MALDI-ToF-MS  | 107 |
| 10.2.4 | Time- and pH-dependent adsorption of chymotrypsin to SiO <sub>2</sub> and TiO <sub>2</sub>  | 108 |
| 10.2.5 | Concentration-dependent adsorption of chymotrypsin to SiO <sub>2</sub> and TiO <sub>2</sub>   | 109 |
| 10.2.6 | Desorption studies  | 110 |

|        |   |     |
|--------|---|-----|
| 10.2.7 | Molecular Dynamics simulations  | 110 |
| 10.3   | Results   | 113 |
| 10.3.1 | Characterization of SiO <sub>2</sub> and TiO <sub>2</sub> colloidal particles   | 113 |
| 10.3.2 | Dependence of the protein adsorption on incubation time and pH  | 117 |
| 10.3.3 | MALDI-ToF-MS analysis of possible autolysis   | 118 |
| 10.3.4 | Analysis of the adsorption layers   | 120 |
| 10.3.5 | Dependence of the adsorbed amount on the protein concentration  | 122 |
| 10.3.6 | Long-range interaction force of chymotrypsin over SiO <sub>2</sub> and TiO <sub>2</sub>   | 123 |
| 10.3.7 | Interaction forces for chymotrypsin multi-layers  | 126 |
| 10.3.8 | Explicit-solvent MD simulations of chymotrypsin adsorption on TiO <sub>2</sub> and SiO <sub>2</sub>   | 128 |
| 10.4   | Discussion and conclusions  | 132 |
| 10.5   | Acknowledgments   | 135 |
| 10.6   | Appendix: Circular dichroism studies  | 135 |
| 10.7   | Source  | 138 |
| 11     | Assessment of the proteolytic activity of $\alpha$ -chymotrypsin immobilized on colloidal particles by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy | 140 |
| 11.1   | Introduction  | 140 |
| 11.2   | Materials and methods   | 142 |
| 11.2.1 | Materials   | 142 |
| 11.2.2 | Characterization of alumina and silica colloidal particles  | 143 |
| 11.2.3 | Chymotrypsin immobilization on alumina and silica colloidal particles   | 144 |
| 11.2.4 | Proteolysis test by MALDI-ToF-MS measurements   | 146 |
| 11.2.5 | Data analysis   | 147 |

|   |     |
|---|-----|
| 11.3 Results  | 148 |
| 11.4 Discussion   | 156 |
| 11.5 Conclusions  | 160 |
| 11.6 Acknowledgments  | 161 |
| 11.7 Appendix: Characterization of colloidal particles after surface functionalization and enzyme immobilization using Fourier transform infrared (FTIR) spectroscopy | 161 |
| 11.8 Source   | 163 |
| 12 Conclusions  | 164 |
| 13 Outlook  | 168 |
| List of students projects   | 170 |
| List of publications  | 171 |
| Selected oral and poster presentations  | 172 |
| 14 References   | 173 |

## List of abbreviations and symbols

### *Abbreviations:*

|                                    |  |
|------------------------------------|--|
| AFM                                | Atomic force microscopy  |
| Al <sub>2</sub> O <sub>3</sub>     | $\alpha$ -Al <sub>2</sub> O <sub>3</sub> , alumina                           |
| Al-OH                              | Hydroxyl groups on alumina surface   |
| APBS                               | Adaptive Poisson-Boltzmann Solver  |
| Asp                                | Aspartic acid  |
| BCA                                | Bicinchoninic acid   |
| BCA assay                          | Bicinchoninic acid protein assay   |
| BET equation                       | Brunauer, Emmet, and Teller equation   |
| CD                                 | Circular dichroism   |
| -COOH                              | Carboxyl group   |
| Cu <sup>2+</sup> , Cu <sup>+</sup> | Cuprous cations  |
| CVD                                | Chemical vapor deposition  |
| CVI                                | Colloidal vibration current  |
| d <sub>50</sub>                    | Median of particle size  |
| Da                                 | Dalton, atomic mass unit (1 Da = 1 g mol <sup>-1</sup> )                     |
| ddH <sub>2</sub> O                 | Double deionized water   |
| DLS                                | Dynamic light scattering   |
| DLVO theory                        | Derjaguin, Landau, Verwey, and Overbeck theory                               |
| DSSP                               | Define Secondary Structure of Proteins method                                |
| FT-IR                              | Fourier transform infrared spectroscopy                                      |
| GRCEL                              | Glycylarginylcysteinylglutamylleucine  |
| HCl                                | Hydrochloric acid  |
| HR-TEM                             | High-resolution transmission electron microscopy                             |
| E                                  | Enzyme   |
| ES                                 | Enzyme-substrate complex   |
| His                                | Histidine  |
| IEP                                | Isoelectric point  |
| ITC                                | Isothermal titration calorimetry   |
| KBr                                | Potassium bromide  |
| KOH                                | Potassium hydroxide  |
| LEaP                               | Link, Edit and Parm program  |
| LINCS                              | 'Linear constraint solver' algorithm   |
| MALDI-ToF-MS                       | Matrix-assisted laser desorption ionization time-of-flight mass spectroscopy |
| MD                                 | Molecular Dynamics simulations   |
| MRSL                               | Methionylarginylserinylleucine   |

|                    |   |
|--------------------|---|
| MRW                | Mean residue weight                                       |
| MS                 | Mass spectroscopy   |
| MUSIC              | Multi site complexation model                             |
| -NH <sub>2</sub>   | Amino group   |
| NMR                | Nuclear magnetic resonance spectroscopy                   |
| -OH                | Hydroxyl group  |
| P                  | Product   |
| PAA <sub>SEM</sub> | Theoretical protein accessible area based on SEM images   |
| PDB                | Brookhaven Protein Database                               |
| PZC                | Point of zero charge                                      |
| RDF                | Radial distribution function                              |
| RSA                | Random sequential adsorption                              |
| S                  | Substrate   |
| SD                 | Standard deviation  |
| SDS-PAGE           | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SEM                | Scanning electron microscopy                              |
| Ser                | Serine  |
| SiO <sub>2</sub>   | Silicon dioxide, silica                                   |
| SPR                | Surface Plasmon resonance                                 |
| SSA <sub>BET</sub> | Specific surface area calculated using BET equation       |
| TEM                | Transmission electron microscopy                          |
| TIP3P              | Water model   |
| TiO <sub>2</sub>   | Titanium dioxide, titania                                 |
| UV-Vis             | Ultraviolet-visible                                       |
| VMD                | Visual Molecular Dynamics                                 |

*Symbols:*

|                |   |
|----------------|---|
| Å              | Ångström, 1 Å = 0.1 nm  |
| A              | Absorption in Lambert-Beer law  |
| K <sub>a</sub> | Acid dissociation constant  |
| Z <sub>S</sub> | Acoustic impedances of the suspension   |
| Z <sub>T</sub> | Acoustic impedances of the transducer   |
| A (ω)          | Acoustic wave frequency   |
| N              | Avogadro's number (6.02214129 * 10 <sup>23</sup> mol <sup>-1</sup> )                                  |
| K <sub>B</sub> | Boltzmann constant (1.3806488 * 10 <sup>-23</sup> m <sup>2</sup> kg s <sup>-2</sup> K <sup>-1</sup> ) |

|                        |   |
|------------------------|---|
| $\psi$                 | Bond between the $\alpha$ -carbon and the carbonyl carbon in proteins |
| $\Phi$                 | Bond between the nitrogen and the $\alpha$ -carbon in proteins        |
| $x, y, \text{ and } z$ | Cartesian coordinates   |
| $[E_{\text{free}}]$    | Concentration of free enzyme  |
| $c_i^0$                | Concentration of ion $i$ in the bulk medium                           |
| $c'$                   | Concentration of the optically active substance                       |
| $\theta$               | Coverage of the surface with proteins                                 |
| $\alpha$               | Cross-sectional area of a gas nitrogen molecule                       |
| $x_D$                  | Debye-Huckel length   |
| $\rho$                 | Density   |
| $\rho_m$               | Density of the medium   |
| $\rho_p$               | Density of the particles  |
| $K_d$                  | Dissociation constant   |
| $x$                    | Distance from particle surface  |
| $W_{\text{DLVO}}$      | DLVO interaction energy   |
| $\mu_D$                | Dynamic electrophoretic mobility of the particles                     |
| $I_n$                  | Electric current, which compensates for $I_s$                         |
| $K^\infty$             | Electrical conductivity of the medium                                 |
| $W_{\text{DL}}$        | Electrostatic interaction energy                                      |
| $\Psi$                 | Electrostatic potential at a given position in the diffuse layer      |
| $e$                    | Elementary charge   |
| $\Theta$               | Ellipticity   |
| $H$                    | Enthalpy  |
| $S$                    | Entropy   |
| $K$                    | Equilibrium constant  |
| $E$                    | Extinction  |
| $\varepsilon$          | Extinction coefficient  |
| $S_0$                  | Free adsorption sites   |
| $M$                    | Frequency of the acoustic wave  |
| $p$                    | Gas pressure  |
| $G$                    | Gibbs energy  |
| $A$                    | Hamaker constant  |
| $h$                    | Hour  |
| $v_0$                  | Initial velocity  |
| $I_0$                  | Intensity of incident light   |
| $I$                    | Intensity of transmitted light  |
| $I$                    | Ionic strength  |
| $\nu$                  | Kinematic viscosity   |

|                         |   |
|-------------------------|---|
| $K_L$                   | Langmuir constant   |
| $c_i$                   | Local ion density   |
| $p_e$                   | Local electric charge density in the diffuse layer                                      |
| $\beta_{ii}$            | London-van der Waals constant   |
| $W_{vdW}$               | London-van der Waals interaction potential  |
| $m/z$                   | Mass-to-charge ratio  |
| $\Gamma_{max}$          | Maximal adsorbed protein amount   |
| $V_{max}$               | Maximum velocity of the reaction  |
| $[\Theta]_{MRW}$        | Mean molar ellipticity  |
| $K_M$                   | Michaelis-Menten constant   |
| $[\Theta]$              | Molar ellipticity   |
| $M$                     | Molecular weight of the adsorbate   |
| $pK_a$                  | Negative decimal logarithm of the acid dissociation constant                            |
| $S_{ads}$               | Number of adsorption sites occupied with proteins or the adsorbed protein concentration |
| $n_i$                   | Number of atoms per unit volume   |
| $S_B$                   | Number of binding sites   |
| $v$                     | Orbit frequency of the electron   |
| $r_1, r_2$              | Particle radii  |
| $R_1, R_2$              | Particle radius   |
| $\Psi_0$                | Particle surface potential  |
| $\phi$                  | Particle volume fraction  |
| $H$                     | Particles' separation distance  |
| $l$                     | Pathlength  |
| $\epsilon_0$            | Permittivity of free space ( $8.854187 \cdot 10^{-12} \text{ F m}^{-1}$ )               |
| $pH_{PZC}$              | pH of point of zero charge  |
| $n$                     | $\pi$   |
| $h$                     | Planck constant ( $6.62606957 \cdot 10^{-34} \text{ m}^2 \text{ kg s}^{-1}$ )           |
| $\alpha$                | Polarizability of the material  |
| $c$                     | Protein concentration in solution   |
| $[H^+]$                 | Proton activity in $\text{mol m}^{-3}$  |
| $r$                     | Radial distance at any point in the double layer from the center of the particle        |
| $R_s$                   | Radius of the spherical particle  |
| $k_{ads}$ and $k_{des}$ | Rate constants for adsorption and desorption  |
| $\kappa$                | Reciprocal Debye-Huckel length  |
| $K$                     | Reciprocal of the Langmuir constant   |
| $\epsilon_r$            | Relative permittivity of the medium   |
| $p_0$                   | Saturation gas pressure   |

|                                  |   |
|----------------------------------|---|
| R                                | Side chain of an amino acid   |
| Si-OH                            | Silanol group   |
| Si-O-Si                          | Siloxane group  |
| [ $\Psi$ ]                       | Specific ellipticity  |
| $\Delta_{ads} G^0$               | Standard Gibbs energy of adsorption   |
| [S]                              | Substrate concentration   |
| $\sigma_0$                       | Surface charge density  |
| [MOH], $MO^-$ ,<br>[ $MOH_2^+$ ] | Surface concentrations of the corresponding surface groups                      |
| $K_S$                            | Surface conductivity of the double layer  |
| $I_S$                            | Surface current   |
| $N_S$                            | Surface density of reactive sites (sites per $m^2$ )                            |
| T                                | Temperature   |
| t                                | Time  |
| [ $E_{total}$ ]                  | Total enzyme concentration  |
| $k_{cat}$                        | Turnover number of the enzyme   |
| $z_i$                            | Valence of the ion i  |
| $\eta$                           | Viscosity   |
| $V_m$                            | Volume of gas building a monolayer on the particle surface                      |
| $W_i$                            | Work required to bring the $i$ th ion to the position with the potential $\Psi$ |
| $\zeta$                          | Zeta potential  |
| $\Psi_\zeta$                     | Zeta potential of particles after protein adsorption                            |

## Summary

The aim of this study was to investigate the effects of adsorption on ceramic particles on the catalytic activities of enzymes. Although the key interaction forces that govern protein adsorption on inorganic particles are known, their influence on enzymatic activity has been poorly evaluated and difficult to predict. Enzyme immobilization on inorganic oxide particles is a widely employed technique that permits the reuse of costly enzymes in catalytic processes. An understanding of the major factors that control protein adsorption and their impact on the catalytic activities of enzymes will lead to more individualized immobilization with increased enzymatic activity after immobilization.

The adsorption of the proteolytic enzyme  $\alpha$ -chymotrypsin on silica ( $\text{SiO}_2$ ), alumina ( $\text{Al}_2\text{O}_3$ ), and two types of titania ( $\text{TiO}_2$ ) was studied. The enzyme adsorption process was specifically investigated by extensive material characterization before and after adsorption, quantification of the adsorbed enzyme and detailed enzymatic activity measurements. The assays required to investigate the enzymatic activity of the adsorbed chymotrypsin were optimized and adapted for specific application to oxide colloidal particles based on known assays for dissolved enzymes. Furthermore, the experimental results were interpreted based on complementary simulations. Covalent enzyme immobilization on amino-functionalized  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$  was also performed. The effects of immobilization on the enzymatic activity of  $\alpha$ -chymotrypsin were additionally investigated by employing matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-ToF-MS) using lysozyme as the enzyme substrate to analyze multiple lysozyme-derived peptides after proteolytic digestion. The main findings of this thesis are as follows:

### *Physisorption:*

- Chymotrypsin adsorbed efficiently on all tested colloidal particles in a concentration- and pH-dependent manner; adsorption increased with increasing pH.
- Adsorption increased as the hydrophobicity of the particles increased.
- The highest adsorption affinities were exhibited by the two types of  $\text{TiO}_2$ , followed by  $\text{Al}_2\text{O}_3$  and then  $\text{SiO}_2$ .

- An activity assay for adsorbed enzymes was established as a simple, rapid method to determine the changes in the catalytic activities of adsorbed enzymes.
- A substantial loss in enzymatic activity was observed after adsorption for all tested colloidal particles.
- For chymotrypsin adsorbed on TiO<sub>2</sub> (rutile), an increase in the K<sub>M</sub> value for the artificial substrate p-nitrophenyl acetate (p-NPA) was analyzed and potentially attributed to blockage of the active site or conformational changes due to adsorption.
- Lateral enzyme-enzyme interactions appeared to have little influence on enzymatic activity, which was similar for all colloidal particles and was largely independent of the surface density of the adsorbed enzymes.

#### *Covalent immobilization:*

- The formation of lysozyme-derived peptides was induced by covalently immobilized chymotrypsin on the surface of particles.
- Kinetic studies revealed that chymotrypsin bound to colloidal particles remained active longer than unbound chymotrypsin.
- A reduction in enzymatic activity and slower digestion kinetics were observed after immobilization to both Al<sub>2</sub>O<sub>3</sub> and SiO<sub>2</sub> particle types.
- The loss of enzymatic activity was more pronounced for SiO<sub>2</sub> than for Al<sub>2</sub>O<sub>3</sub> in cyclic reusability studies, likely due to differences in the digestion reaction and possible steric hindrance.
- Both materials retained lysozyme digestion activity after 7 weeks of storage at room temperature.

## Zusammenfassung

Das Ziel dieser Arbeit war die Untersuchung der Auswirkungen der Enzymadsorption auf keramischen Partikeln auf die katalytische Aktivität der Enzyme. Obwohl die wichtigen Wechselwirkungen, die die Proteinadsorption auf Oxidpartikeln beeinflussen, bekannt sind, ihr Einfluss auf die enzymatische Aktivität ist wenig untersucht und unzureichend vorhersehbar. Enzymimmobilisierung auf anorganischen Oxidpartikeln ist eine weit verbreitete Methode um die Wiederverwendbarkeit der kostspieligen Enzyme in katalytischen Prozessen zu ermöglichen. Das Verstehen der Hauptfaktoren, die die Adsorption steuern und deren Einfluss auf die katalytische Aktivität der Enzyme kann zur Auswahl einer individuelleren Immobilisierungsmethode und somit zu einer gesteigerten enzymatischen Aktivität nach der Immobilisierung führen.

Die Adsorption des proteolytischen Enzyms  $\alpha$ -Chymotrypsin auf Siliciumoxid ( $\text{SiO}_2$ ), Aluminiumoxid ( $\text{Al}_2\text{O}_3$ ) und auf zwei Arten von Titanoxid ( $\text{TiO}_2$ ) wurde erforscht. Der Prozess der Enzymadsorption wurde mit Hilfe der ausführlichen Materialcharakterisierung vor und nach der Adsorption, der Bestimmung der adsorbierten Enzymmenge und der detaillierten Messungen der enzymatischen Aktivität untersucht. Die Analysemethoden, die für die Untersuchung der katalytischen Aktivität des adsorbierten Chymotrypsin nötig waren, wurden im Rahmen dieser Arbeit für die spezielle Anwendung mit kolloidalen Oxidpartikeln basierend auf bekannter Untersuchungsmethode für gelöste Enzyme optimiert und weiterentwickelt. Desweiteren wurden die experimentellen Ergebnisse basierend auf ergänzenden Simulationen interpretiert. Zusätzlich wurde die Methode der kovalenten Enzymimmobilisierung auf aminofunktionalisierten  $\text{Al}_2\text{O}_3$ - und  $\text{SiO}_2$ -Partikeln angewendet. Die Auswirkung auf die katalytische Aktivität von  $\alpha$ -Chymotrypsin wurde mit MALDI-ToF-MS untersucht, indem die Peptide aus dem proteolytischen Verdau des Substrates Lysozym analysiert wurden. Die Hauptergebnisse dieser Arbeit sind folgende:

### *Physisorption:*

- Chymotrypsin adsorbierte auf allen getesteten kolloidalen Partikeln und wies dabei Konzentrations- und pH-Abhängigkeit auf, wobei die Adsorption mit steigendem pH gestiegen ist.
- Höhere Adsorption wurde für hydrophobere Oberflächen beobachtet.

- Beide Arten von  $\text{TiO}_2$  zeigten die höchste Adsorptionsaffinität, gefolgt von  $\text{Al}_2\text{O}_3$  und zuletzt von  $\text{SiO}_2$ .
- Aktivitätstest für adsorbierte Enzyme wurde etabliert und diente als einfache und schnelle Methode zur Bestimmung der Änderungen der katalytischen Aktivität von adsorbierten Enzymen.
- Erheblicher Verlust der enzymatischen Aktivität wurde nach der Adsorption auf allen getesteten kolloidalen Partikeln beobachtet.
- Für das adsorbierte Chymotrypsin auf  $\text{TiO}_2$  (Rutil): Anstieg des  $K_M$  Wertes für das künstliche Substrat p-Nitrophenylacetat (p-NPA) wurde festgestellt, wobei die Blockierung des Aktivitätszentrums oder die Konformationsänderungen wahrscheinliche Ursachen dafür waren.
- Seitliche Wechselwirkungen der benachbarten adsorbierten Enzyme schienen die enzymatische Aktivität kaum zu beeinflussen, da diese für alle kolloidalen Partikel größtenteils unabhängig von der Dichte der adsorbierten Enzyme auf der Oberfläche blieb.

### *Kovalente Immobilisierung:*

- Die Entstehung der Peptide, die Lysozym zugeordnet wurden, wurde durch die Präsenz des kovalent immobilisierten Chymotrypsins auf der Partikeloberfläche herbeigeführt.
- Kinetische Untersuchungen: Chymotrypsin, das auf Partikeln immobilisiert wurde, blieb länger aktiv als das nicht immobilisierte Chymotrypsin.
- Verringerung der enzymatischen Aktivität und langsamere Kinetik der Verdauung wurden nach der Immobilisierung auf  $\text{Al}_2\text{O}_3$  und  $\text{SiO}_2$  beobachtet.
- Die Verringerung der enzymatischen Aktivität bei den Wiederverwendungsstudien war mehr ausgeprägt für  $\text{SiO}_2$  als für  $\text{Al}_2\text{O}_3$  und war am wahrscheinlichsten durch die Verdaureaktion und die möglichen sterischen Hinderungen zu erklären.

Beide Materialien waren aktiv bei dem Verdau von Lysozym nach 7 Wochen Lagerung bei Raumtemperatur.

