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Dipl.-Chem. Ludmilla Derr, Bremen

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

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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 α-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 α-chymotrypsin were additionally investigated by employing matrix-assisted laser desorption ionization time-of-flight mass spectroscopy.

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Ludmilla

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

Søren Kierkegaard

To M. F. C.

Contents

List of abbreviations and symbols

Abbreviations:

AFM	Atomic force microscopy
Al_2O_3	α-Al_2O_3, 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^{2+}, Cu^{+}	Cuprous cations
CVD	Chemical vapor deposition
CVI	Colloidal vibration current
d_{50}	Median of particle size
Da	Dalton, atomic mass unit (1 Da = 1 g mol^{-1})
ddH_2O	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
$-NH_2$	Amino group
NMR	Nuclear magnetic resonance spectroscopy
-OH	Hydroxyl group
P	Product
PAA_{SEM}	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_2	Silicon dioxide, silica
SPR	Surface Plasmon resonance
SSA_{BET}	Specific surface area calculated using BET equation
TEM	Transmission electron microscopy
TIP3P	Water model
TiO_2	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_a	Acid dissociation constant
Z_S	Acoustic impedances of the suspension
Z_T	Acoustic impedances of the transducer
A (ω)	Acoustic wave frequency
N	Avogadro's number ($6.02214129 * 10^{23}$ mol^{-1})
k_B	Boltzmann constant ($1.3806488 * 10^{-23}$ m^2 kg s^{-2} K^{-1})

ψ	Bond between the α-carbon and the carbonyl carbon in proteins
Φ	Bond between the nitrogen and the α-carbon in proteins
x, y, and z	Cartesian coordinates
$[E_{free}]$	Concentration of free enzyme
c_i^0	Concentration of ion i in the bulk medium
c′	Concentration of the optically active substance
θ	Coverage of the surface with proteins
α	Cross-sectional area of a gas nitrogen molecule
x_D	Debye-Huckel length
ρ	Density
ρ_m	Density of the medium
ρ_p	Density of the particles
K_d	Dissociation constant
x	Distance from particle surface
W_{DLVO}	DLVO interaction energy
μ_D	Dynamic electrophoretic mobility of the particles
I_n	Electric current, which compensates for I_s
K^∞	Electrical conductivity of the medium
W_{DL}	Electrostatic interaction energy
ψ	Electrostatic potential at a given position in the diffuse layer
e	Elementary charge
Θ	Ellipticity
H	Enthalpy
S	Entropy
K	Equilibrium constant
E	Extinction
ε	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
v	Kinematic viscosity

K_L	Langmuir constant
c_i	Local ion density
p_e	Local electric charge density in the diffuse layer
β_{ii}	London-van der Waals constant
W_{vdW}	London-van der Waals interaction potential
m/ z	Mass-to-charge ratio
Γ_{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
Ψ_0	Particle surface potential
φ	Particle volume fraction
H	Particles' separation distance
l	Pathlength
ε_0	Permittivity of free space ($8.854187 * 10^{-12}$ F m^{-1})
pH_{PZC}	pH of point of zero charge
n	Pi
h	Planck constant ($6.62606957 * 10^{-34}$ m^2 kg s^{-1})
α	Polarizability of the material
c	Protein concentration in solution
$[H^+]$	Proton activity in 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
κ	Reciprocal Debye-Huckel length
K	Reciprocal of the Langmuir constant
ε_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
[Ψ]	Specific ellipticity
$\Delta_{ads} G^0$	Standard Gibbs energy of adsorption
[S]	Substrate concentration
σ_0	Surface charge density
[MOH], MO^-], $[MOH_2^+]$	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
η	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 Ψ
ζ	Zeta potential
Ψ_ζ	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 α-chymotrypsin on silica (SiO_2), alumina (Al_2O_3), and two types of titania (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 Al_2O_3 and SiO_2 was also performed. The effects of immobilization on the enzymatic activity of α-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 TiO_2, followed by Al_2O_3 and then 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_2 (rutile), an increase in the K_M 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_2O_3 and SiO_2 particle types.
- The loss of enzymatic activity was more pronounced for SiO_2 than for Al_2O_3 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 α-Chymotrypsin auf Siliciumoxid (SiO_2), Aluminiumoxid (Al_2O_3) und auf zwei Arten von Titanoxid (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 Al_2O_3- und SiO_2-Partikeln angewendet. Die Auswirkung auf die katalytische Aktivität von α-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 TiO_2 zeigten die höchste Adsorptionsaffinität, gefolgt von Al_2O_3 und zuletzt von 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 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 Al_2O_3 und SiO_2 beobachtet.
- Die Verringerung der enzymatischen Aktivität bei den Wiederverwendungsstudien war mehr ausgeprägt für SiO_2 als für Al_2O_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.