Stabilization of Industry-Relevant Enzyme Formulations

Stabilisierung von Industrie-relevanten Enzymformulierungen

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“Laundry is the only thing that should be separated by color.”

Author unkown

Dissemination of Research Work

List of Publications and Patents

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Reinoso Garcia, M.; Baier, G.; Kübelbeck, S.; Date of the Invention: 08.03.2018. Invention Status: Evaluation.

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Functional Enzyme Carriers; Baier, G.; Kübelbeck, S.; Konradi, R.; Karwacki, L.; Treuling, U.; Treier-Marxen, K.; CARE 2018; 25.01.2018; Ludwigshafen, Germany.

Correlation of enzyme shelf life and differential scanning calorimetry as predictive tool to evaluate enzyme stability in liquid detergent formulation; Kübelbeck, S.; Lan, Y.; Andre, A.; Baier, G.; 3rd BASF Global Analytics Conference; 16.-17.05.2018; Ludwigshafen, Germany.

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Evaluation Competency to utilize Metal-Organic Frameworks (MOFs) for Enzyme Application; Kübelbeck, S.; Baier, G.; Treuling, U.; Speros, J.; Ji, Z.; Yaghi, O.; CARE 2019; 24.01.2019; Ludwigshafen, Germany.

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Table of Contents

1. Abstract – Zusammenfassung

What do all enzyme-containing liquid detergents have in common, besides their diversity? At the time of washing large parts of all enzymes are already inactive and denatured. Accordingly, the washing performance of the detergent is strongly reduced. There are various causes which lead to this undesirable loss of function. Starting with the presence of surfactants and chelating agents, proteolytic degradation as well as incorrect storage conditions. Known solution approaches focus on the temporal inhibition of protease and neglect the other denaturing factors and enzymes. This thesis deals with four different strategies to stabilize more than just one detergent enzyme against surfactants as well as against proteolysis. Enzyme stabilities are valued by measuring enzyme activity in storage tests and by determining thermal stability of the enzymes in a single measurement. In this context a good correlation between both methods has been identified. This correlation enables a long-term enzyme activity prediction based on one thermal stability measurement. Consequently, the number of time-consuming long-term storage tests can be reduced.

Firstly, small molecules interacting with the enzyme´s active site are tested to enhance the enzyme stability under detergent conditions. Remedial measures to increase the stability of enzymes usually target protease, the most relevant detergent enzyme and a main reason for the inactivation of all detergent enzymes including itself. Here, another enzyme, lipase, has been chosen as starting point. Lipase plays a decisive role in stain removal and is very sensitive to proteolysis. It is presumed that a substrate (acetylcholine) from a related enzyme (acetylcholinesterase) can interact as a competitive inhibitor. Along this route acetylcholine and three derivatives are tested with respect to enzyme stabilization and the stability of lipase is significantly increased over a storage period of four weeks in a standard detergent formulation at elevated temperatures.

Comparatively, stabilization effects have been obtained through the synthesis of enzyme-polymer conjugates – the second strategy. Polysaccharides and polyethylene glycol with amino reactive groups have been covalently grafted to protease, α-amylase and lipase. Polysaccharides in case of α-amylase and especially polyethylene glycol for lipase deliver the best results.

The last two strategies focus on the encapsulation and immobilization of lipase. Lipase appears to be the most suitable detergent enzyme for those two strategies due to a positive effect on lipase activity by hydrophobic environments. Silica nanoparticles are synthesized in the first step and mixed with the enzyme afterwards. Due to the unfavorable pK_a value and size of the detergent lipase, it is difficult to immobilize the enzyme into the pores of the pre-synthesized mesoporous silica nanoparticles. As a second immobilization system metal-organic frameworks have been examined. In general, metal-organic frameworks possess smaller pores than silica nanoparticles. Accordingly, the framework

is built up around the enzyme *in-situ* and the diffusion step is ceased. Lipase embedded into MIL-53 shows an increased stability in a standard detergent formulation as well as against proteolysis.

Was haben alle Flüssigwaschmittel, unabhängig von ihrer Vielfalt, gemeinsam? Zum Zeitpunkt, wenn der Endnutzer seine Waschmaschine startet, sind große Teile der Enzyme im Waschmittel bereits denaturiert und dadurch inaktiv. Zu diesem Funktionsverlust tragen mehrere Faktoren bei: Das Vorhandensein von Tensiden und Chelatbildnern, proteolytischer Abbau und unpassende Lagerbedingungen (Temperatur). Die bekannten Lösungsansätze fokussieren auf die temporäre Inhibierung der Protease und vernachlässigen die anderen vorhandenen Faktoren, die zur Denaturierung führen. Außerdem werden die Waschmittelenzyme neben der Protease nicht in die Stabilisierung mit einbezogen. Die vorliegende Arbeit diskutiert vier Strategien, mit dem Ziel mehr als nur ein Waschmittelenzym gegenüber den Tensiden und der Protease zu stabilisieren. Dabei wird die Enzymstabilität anhand von zwei unterschiedlichen Methoden bewertet: Messungen der Enzymaktivität in Lagerversuchen und Bestimmung der thermischen Stabilität von Enzymen in einer einzelnen Messung. In diesem Zusammenhang ist eine gute Korrelation zwischen beiden Methoden entdeckt worden. Die Korrelation ermöglicht eine langfristige Vorhersage der Enzymstabilität auf Grundlage einer Messung zur thermischen Stabilität des Enzyms. Zukünftig ist es damit möglich, die Anzahl an zeitaufwendigen Langzeitlagerversuchen zu reduzieren.

Zunächst werden kleine Moleküle, die mit dem aktiven Zentrum eines Enzyms wechselwirken können, zur Verbesserung der Stabilität getestet. Bisher bekannte Maßnahmen zur Stabilitätsverbesserung von Enzymen in Waschmitteln beziehen sich alle auf Protease, da diese das am häufigsten eingesetzte Flüssigwaschmittelenzym darstellt und mit ein Hauptgrund für den Aktivitätsverlust aller Enzyme ist. Hier ist mit Lipase ein anderes Enzym als Ausgangspunkt gewählt. Lipase spielt eine entscheidende Rolle in der Fleckenentfernung und ist selbst sehr anfällig gegenüber Proteolyse. Hintergrund der ausgesuchten kleinen Moleküle ist, dass ein Substrat (Acetylcholin) von einem der Lipase verwandten Enzym (Acetylcholinesterase) bei Lipase selbst als kompetitiver Inhibitor wirken kann. Auf diesem Weg sind neben Acetylcholin drei weitere Derivate getestet worden und es ist möglich gewesen, die Lipasestabilität während einer vier wöchigen Lagerung in einer Standardwaschmittelformulierung bei erhöhter Temperatur signifikant zu erhöhen.

Ähnliche stabilisierende Effekte sind mit der zweiten Strategie, der Herstellung von Enzym-Polymer-Konjugaten erhalten worden. Polysaccharide und Polyethylenglykol mit Amino-reaktiven Gruppen sind hierbei kovalent an Protease, α-Amylase und Lipase angebunden

worden. Im Fall von α-Amylase liefern Polysaccharide und für Lipase Polyethylenglykol die besten Ergebnisse.

Die letzten beiden Strategien untersuchen die Einkapselung und Immobilisierung von Lipase. Lipase erscheint als vielversprechendstes Waschmittelenzym für diese beiden Strategien, da die Gegenwart eines hydrophoben Materials einen positiven Einfluss auf die Lipaseaktivität haben kann. Mesoporöse Silica-Nanopartikel sind in einem ersten Schritt hergestellt und erst anschließend mit Enzym inkubiert worden. Aufgrund des ungünstigen pK_a-Wertes und der Größe der Waschmittellipase ist es schwierig dieses Enzym in den Poren von zuvor hergestellten Nanopartikeln zu immobilisieren. Als zweite Immobilisierungssystem sind Metallorganische Gerüste untersucht worden. Generell besitzen diese Gerüste kleinere Poren als Silica-Nanopartikel. Dementsprechend ist das Gerüst *in situ* um das Enzym herum gebaut worden und der schwierige Diffusionsschritt konnte umgangen werden. Lipase eingebettet in MIL-53 zeigt eine erhöhte Stabilität in einer Standardwaschmittelformulierung und gegenüber Proteolyse.

2. Introduction and Literature Review

This section summarizes the theoretical part of the present thesis and includes the current state of literature regarding the discussed topics. In the first place, laundry detergents in general and their single components are introduced. Afterwards, it is focused on enzymes – especially protease, α-amylase and lipase. Accordingly, available enzyme stabilization strategies are described and finally, the four strategies used in the present thesis (enzyme-polymer conjugates, small molecules, metal-organic frameworks and mesoporous silica systems) are outlined and discussed.

2.1. Laundry Detergents

Nowadays the market in Europe, America, Australia or Asia is flooded with a large variety of different laundry detergents. In Germany more than 100 detergents for laundry applications are commercially available.[1] These products can be distinguished between their different application fields and thereto relating their chemical composition. The composition varies from country to country, nevertheless, it is possible to define four main types of laundry detergents: heavy-duty and low-duty detergents, color and special detergents. In general, detergents consist of a mixture of four different basic elements, that are described in detail in the following chapters: surfactants, builders, bleaching agents and other ingredients. The central compound of detergents are surfactants that are as well as builders present in all detergent types. Bleaching agents are only added to powder heavy-duty detergents.[1]

A further classification regarding the packaging is possible. Powders have been the first detergents, starting 1907 with Persil®. First liquid detergents entered the market in Germany in 1981 and in 2012 Procter & Gamble introduced laundry detergent pods.[1] The three types of packaging are characterized by various benefits. Using a liquid instead of a powder detergent avoids solubility problems and supports lower washing temperatures. Thereby energy is saved, which enhances the appeal of a liquid detergent to the consumer.[2] In addition, liquid detergents are more gentle to textiles than powders due to the forego of bleaching agents.[1] With regard to the performance powder detergents score better, because of the low stability of enzymes in liquid detergent formulations, whereas in powders a physical separation of enzymes and detrimental compounds is given, leading to a higher enzyme shelf-life.[1-2] Besides the advantages of liquid detergents with pods the dosage for a washing cycle is convenient and the risk for spillage low. The enzyme stability obstacle of regular liquid detergents can be overcome by using multi-chamber pods that allow a spatial separation of enzymes and detrimental compounds like surfactants. However, it is necessary to disrupt the dissolvable packet (typically polyvinyl alcohol) to release the detergent.[1] The instability of enzymes in liquid detergent formulations is taken into account in the present thesis.

Powder detergents dominated the market for years, but in the meantime the consumer preferences for liquid detergents emerged, so that in 2016 liquid detergents achieve a market share of about 49% in Germany (figure 2-1[3]). The relatively newly launched detergent pods reach a market share in Germany of 2%, but in other countries like France or Great Britain the demand is higher (10%). In figure 2-1 the market share 2016 of the three different detergent packing types in France, Russia and Germany is shown. The distribution of the consumer preferences varies widely between France and Russia. While in Russia the laundry is done with powder detergents, are in France liquid detergents most popular. Germany represents the golden mean with a balanced relation of powder to liquid detergent. Detergent pods represent in all three countries the smallest quantity (figure 2-1).[1, 3-4]

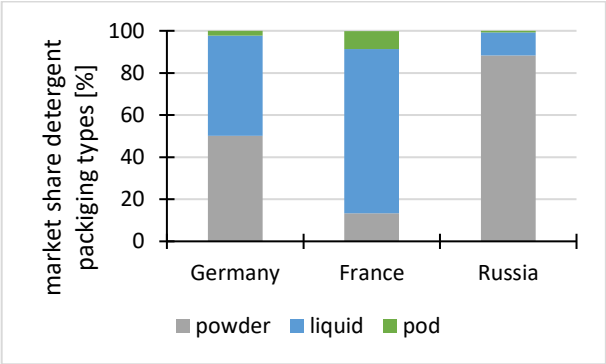


Figure 2-1: Market share of detergent packaging types in Germany, France and Russia in 2016.

In general, the trend is moving in the direction of liquid detergents and relating thereto the demand for an effective enzyme stabilizing system is increasing. To work out an appropriate enzyme stabilization strategy it is necessary to grapple with the composition of liquid detergents. The single compounds are introduced in the following chapters.

2.1.1. Surfactants

Surfactants are the central ingredient of laundry detergents and have been synthesized for this application since the two World Wars. The amphiphilic substances consist of a hydrophobic part, which is composed of long-chain hydrocarbons, and a hydrophilic part that differs depending on the character of the surfactant. Regarding the charge of the hydrophilic part of a surfactant a distinction between anionic, cationic, nonionic and amphoteric is made. The major part of surfactants in detergents is formed by linear alkylbenzene sulfonates (LAS; figure 2-2 a)), fatty alcohol ether sulfates (FAES; figure 2-2 b)) and fatty alcohol ethoxylates (FAEO; figure 2-2 c)). The first two groups belong to anionic surfactants, whereas the last is part of nonionic surfactants. In figure 2-2 the chemical

structures of those surfactants are displayed. It should be noted that LAS typifies a mixture of isomers and homologues, which are a result of the chemical synthesis.[1, 5-6]

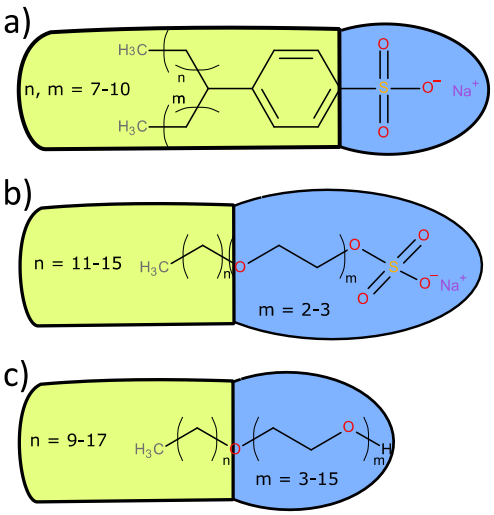


Figure 2-2: Structures of a) linear alkylbenzene sulfonates (LAS), b) fatty alcohol ether sulfates (FAES) and c) fatty alcohol ethoxylates (FAEO). Hydrophilic parts are displayed in blue, hydrophobic parts are shown in yellow.

Due to a cost-effective manufacture, a great water solubility and a good foaming behavior, LAS are the most important criteria for stain removal laundry surfactants. The disadvantages are a sensitivity to hard water and a strong detrimental effect on enzymes. FAES and FAEO are more robust to hard water and less detrimental to enzymes as well as to human skin.[1]

In the washing process many properties of surfactants collaborate to remove stain and to inhibit a new accumulation. The amphiphilic structure of surfactants enables a lowering of the water´s surface tension and the formation of micelles. Introduction of air into an aqueous surfactant solution can result in the formation of a surfactant bilayer or rather a foam bubble. As a result of the lower surface tension stain and textile fibers are completely wetted and surfactants accumulate on both interfaces. Electrostatic repulsion between identically charged stain and textile layer reduces stain adhesion and enables with the support of mechanical movement the removal of stain from the textile. A renewed accumulation is prevented by the formation of a hydrophilic layer around the stain.[1, 5, 7-8] The process is shown in figure 2-3.

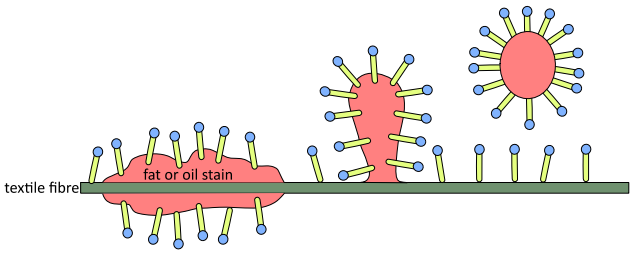


Figure 2-3: Fat or oil stain removal from a textile fiber by surfactants.

Modern laundry detergents consist of a combination of different surfactants. Even surfactants have great washing properties, they cannot clean textiles on their own satisfactory. For example, surfactants alone are ineffective if a stain is hydrophilic, polar and water insoluble such as water based materials like tea or coffee.[1]

For an effective washing process, surfactants are indispensable even if they contribute a major part for enzyme denaturation and inactivation. Since it is not possible to omit surfactants, enzyme stabilization strategies for liquid detergents are necessary.

2.1.2. Builders

It is the task of builders to support the surfactants by reducing the water hardness. Therefore, builders complex calcium, magnesium and other metal ions and prevent a precipitation with the surfactants on the textiles or the washing machine. Regarding the mechanism a distinction is made between precipitation, chelation and ion exchange.[1] The first builders in laundry detergents, sodium silicate and sodium carbonate, have been based on precipitation and the corresponding alkaline earth metal carbonates and silicates have been formed. Thus, on the one hand the washing effect of the surfactants have been increased, but on the other hand the slightly water soluble calcium carbonate is build and the textiles become incrusted.[1] The second generation of builders (1930s) are phosphates and phosphonates with chelating properties. These phosphorous based builders provide buffering properties to stabilize an alkaline pH value and the resulting chelate complexes are water soluble. Nevertheless, the use of phosphates is questionable regarding the environment (eutrophication).[9] Consequently, especially western Europe relies on phosphate-free systems like zeolites. The efficacy of such sodium aluminosilicates is based on the ion exchange of the own sodium ion with other ions of comparable size. But all zeolites are water insoluble, which can lead to a deposit on textiles.[10] Besides other phosphorous-free chelating agents are introduced. These include inter alia copolymers made from acrylic and maleic acid, trisodium citrate or ethylenediaminetetraacetic acid (EDTA).[11]

The fact that some enzymes need metal ions like calcium in their active site to perform well, makes them vulnerable to builders. For this reason, enzymes have to be stabilized against builders.

2.1.3. Bleaching Agents

Bleaching agents are added to powder heavy-duty detergents and are present for colored stains that are resistant to surfactants. In the process organic dyes located in those stains are oxidized and bleached. Thereby, the conjugated double bonds, which are responsible for the color, are fragmented

or hydroxylated to prevent the absorption of visible light and to reduce the adhesion on the textile. Additionally, bleaching agents have a killing effect on microorganisms, so that the hygiene and odor of textiles fibers is increased.[1] Used bleaching agents are sodium perborate or sodium percarbonate as well as sodium hypochlorite. Sodium perborate and percarbonate are applied in Europe especially and are effective through the water-related generation of hydrogen peroxide. Hydrogen peroxide oxidizes the double bounds in a so-called oxygen bleaching.[12] In America sodium hypochlorite is used as bleaching agent among others. Here, a chlorine bleach with hypochlorous acid takes place.[13] Compared with a chlorine bleaching (20 °C) for an effective oxygen bleaching higher washing temperatures (60 °C) are required. To reduce this temperature bleach activators, like tetraacetylethylenediamine (TAED) are added. TAED forms with the generated hydrogen peroxide the strong oxidation agent peracetic acid which enables a decreased washing temperature (30 °C).[14]

Since bleaching agents are not part of liquid detergents, they are not discussed in the further thesis. However, bleach can oxidize amino acids like methionine resulting in an enzyme inactivation.[15]

2.1.4. Further Ingredients

Further ingredients of laundry detergents are enzymes, optical brighteners, corrosion inhibitors, foam inhibitors, anti-redeposition agents, dye transfer inhibitors and fragrances. Detergent enzymes are pointed out more in detail in chapter 2.2. Optical brighteners are fluorescent organic compounds that absorb ultraviolet (UV) light and emit blue light to reduce the yellow tinge of undyed textile fibres.[16] To protect the aluminum components of a washing machine corrosion inhibitors like soluble glass are added.[1] The foam formation is regulated by foam inhibitors[1] and the redeposition of already removed stains is prevented by anti-redeposition agents like carboxymethyl cellulose (CMC).[17-18] Dye transfer inhibitors impede a color transfer from one textile to another[19] and fragrances suppress the odor of the detergent solution and give an own scent to the textiles. Additionally, in powder detergents are fillers like sodium sulfate and in liquid detergents water and alcohols are added.[1]

2.2. Enzymes

If a textile fiber is soiled with dried stains, for example starch-based, surfactants and bleaching agents are insufficient for cleaning, and enzymes are required. Enzymes are biocatalysts made of amino acids that lower the activation energy of chemical reactions. The three-dimensional (3D) folding of the amino acids as well as the resulting conformation of an enzyme and especially of its active binding site is essential for the activity of the biocatalysts. That is why enzymes show a high specificity regarding substrates where only complementary ones can bind and be converted.[20-21] Additionally, enzymes catalyze reactions chemoselectively, regioselectively and stereoselectively, so that undesirable side reactions are suppressed and complex structures can be buildup.[22] However, the conformational stability of enzymes can be disrupted and destroyed by physical or chemical influences that are located outside of the physiological conditions. The loss of the superordinate structure is known as denaturation. There, the order of amino acids remains unchanged but combined with the native folding the enzyme activity is lost. Renaturation is the reverse process, if it is possible to return to the native folding and to regain enzyme activity.[23] Physical denaturing factors include temperature, radiation or mechanical stress. Temperature-induced unfolding is often irreversible and joined with a reduction of solubility.[24] Chemical denaturation can be induced by extreme pH values, chaotropic salts, surfactants or organic solvents.[25]

In general, reactions catalyzed with enzymes are more environmentally friendly due to lower energy costs as well as less harmful chemicals and less waste products.[26] Therefore, enzymes are involved into a broad range of industrial applications such as food, chemicals, medicine or detergent. For those various applications enzymes are specifically designed by protein engineering to show high selectivity, activity and stability.[27-28] This results in a variety of different enzymes from which more than 7,500 are listed in the database BRENDA.[29]

Detergent enzymes make up about 30% of the total worldwide enzyme production and are specific regarding one type of stain or rather one substance class. They often degrade those insoluble macromolecules into small fragments that can be removed from the textile. In 1913 OTTO RÖHM added an enzyme, isolated from porcine pancreas to a laundry detergent for the first time.[9] Nowadays, a mixture of at least five different enzymes is present in about 90% of all laundry detergents.[30] In table 2-1 the detergent enzymes, their substrate and market launch are listed.

Table 2-1: List of five detergent enzymes, their substrates and market launch.

Enzyme	Substrate	Market launch
Protease	Peptide bonds in proteins	1960[1]
Amylase	Glycosidic bonds in starches	1975[1]
Lipase	Ester bonds in fats	1991[21]
Cellulase	Glycosidic bonds in celluloses	1992[1]
Mannanase	Galactomannan	2002[1]

The resulting benefits due to the use of enzymes in laundry detergents are a lower washing temperature and a reduction of the necessary quantity of detergent.[1, 31]

Furthermore, the textile fibers itself are treated more gently during the washing process. For instance, cellulase has additional anti-greying and anti-redeposition properties.[32] In connection with the use of enzymes in laundry detergents some challenges arise. The fine dust of enzymes has a sensitizing effect and causes allergies. For that reason enzymes have been encapsulated for powder detergents since the end of 1960s.[33] So called enzyme prills consisting of a sprayed wax melt with enzyme has been on the market since 1970.[34] For powder detergents this results in the following advantages: on the one hand the human health is protected against sensitizing effects and on the other hand the enzyme itself is isolated from external denaturing influences that are present within a detergent. In this way the storage stability of the enzyme is increased.[34-35] For the application in a liquid detergent this physical separation is not possible. Therefore, other strategies have to be investigated. In the following chapters three enzymes – protease, α-amylase and lipase – are described more in detail.

2.2.1. Protease

With regard to an application in the area of laundry detergents, protease is the most important, most widely and longest used enzyme.[1] Proteases belong to the hydrolases and catalyze the proteolysis of peptide bonds. They are present in all tissues and cells of all organism and are divided into intracellular and extracellular proteases.[36] With regard to the active site they are further subdivided into serine, threonine, cysteine, aspartate, glutamate, asparagine or metallo proteases.[37]

A detergent protease (EC 3.4.21.62) is a nonspecific extracellular alkaline serine endopeptidase expressed from a *Bacillus* strain, often *Bacillus subtilis*.[38] Furthermore, the enzyme is a single-domain monomer and possess a globular 3D structure with a three layer (αβα)-sandwich. Thereby, calcium ions serve as a co-factor.[39]

The eponymous serine (Ser) is in the active site which has the structure of a catalytic triad. This triad is completed by histidine (His) and asparagine (Asp). In figure 2-4 the catalytic mechanism of a

Ser-His-Aps catalytic triad is illustrated. As a first step a nucleophilic attack on the carbonyl group of the peptide bond (= substrate) occurs originating from Ser221 (figure 2-4 A). Therefore, His64 serves as a general acid/base due to its imidazole ring. Whereby, a tetrahedral intermediate state is formed (figure 2-4 B). The released proton is accommodated by the imidazole of the His64. The tetrahedral intermediate state is stabilized by the oxyanion hole, which is formed by the amino groups of the peptide backbone. In a subsequent step, the tetrahedral intermediate state decomposes and an acyl-enzyme intermediate is formed. In that regard, the peptide is cleaved. (figure 2-4 C). As a further step, a water molecule activated by His64 attacks the acyl-enzyme intermediate nucleophilic (figure 2-4 D) and a second tetrahedral intermediate state is formed (figure 2-4 E). Finally, the cleaved substrate is released, and the enzyme is regenerated (figure 2-4 F).[40-41]

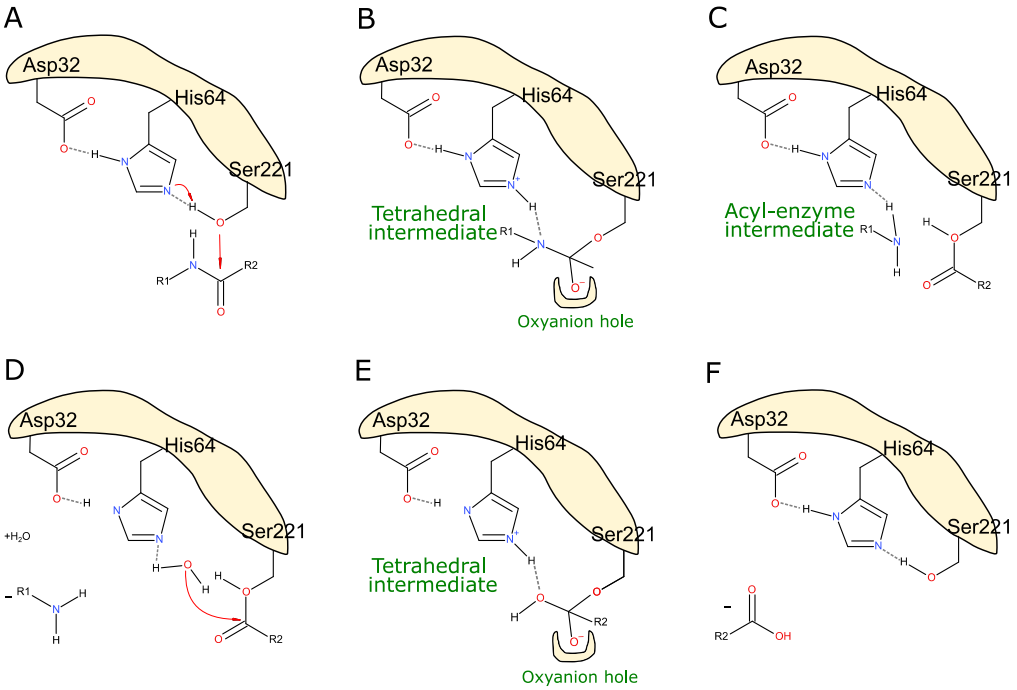


Figure 2-4: Schematic illustration of a Ser-His-Asp catalytical triad. A: beginning state, B: tetrahedral intermediate I, C: acyl-enzyme intermediate, D: acyl-enzyme intermediate, E: tetrahedral intermediate II, F: regenerated enzyme.

2.2.2. α-Amylase

The endoenzyme α-amylase (EC 3.2.1.1) catalyzes the hydrolysis of α-1,4-glycosidic bonds in starch statistically.[42] Thereby, the conformation of the anomeric center remains unchanged[43] and a mixture of maltotriose, maltose, glucose and the main product maltopentaose is obtained.[44] The hydrolysis rate is influenced by the substrate size, which means that eight or nine glucose units are cleaved rapidly. While the degradation of units shorter than maltopentaose occurs slowly. Nevertheless,

α-amylase is present in all living organism.[45] For detergent applications α-amylases are often expressed from *Bacillus licheniformis* due to a higher thermal and alkaline pH stability.[38]

X-ray crystallographic analysis from KLEIN ET AL. have shown that α-amylase consists of three domains. Domain A is the central domain and has a barrel-like structure of a TIM barrel in which eight parallel β-sheets are connected via eight α-helices. Domain A is surrounded by domain B (uneven β-structures) and domain C (Greek key motif). Moreover, a calcium ion as a co-factor is located between domain A and B.[46]

The active site of α-amylases is located between domain A and B and consists of three essential amino acids asparagine (Asp231), glutamic acid (Glu261) and a second asparagine (Asp328). Glu261 acts as a proton donor, Asp231 as a nucleophile and Asp328 increases the pK_a value of Glu261.[43, 46-47] In figure 2-5 the three-step mechanism of the hydrolysis is shown. Firstly, the exocyclic oxygen is protonated by Glu261 and carbon C1 is attacked nucleophilic by Asp231. As a result, the reducing end of the substrate (HOR) split off (figure 2-5 I). Secondly, a water molecule activated by Glu261 causes the hydrolysis of the covalent bond between Asp231 and C1 (figure 2-5 II). Thirdly, the molecule groups regenerate (figure 2-5 III).[43, 48]

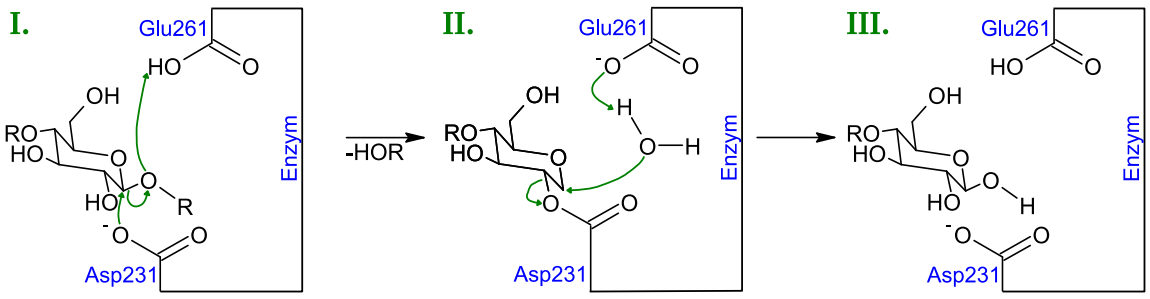


Figure 2-5: Schematic illustration of hydrolysis in an active site of α-amylase. I: nucleophilic attack of Asp231 on C1 of the substrate leads to a cleavage of HOR. II: activated water initiates the hydrolysis of the covalent linkage between Asp231 and C1. III: regeneration of the enzyme.

2.2.3. Lipase

With the combination of surfactant and lipase the washing performance regarding fatty stains is significantly increased. Lipase initiates the dissolution and the surfactants can remove the grease stains easily.[1] Lipases are esterases and catalyze the hydrolysis of long-chain triacylglycerol to di- and monoglycerides as well as glycerol and free fatty acids.[49] Furthermore, lipase shows a broad pH and temperature stability and the presence of a co-factor is not required.[27, 39]

Usually, lipase for detergent applications is expressed from the fungi *Humicola insolens* or *Aspergillus oryzae*.[38] Another option is *Thermomyces lanuginosus*, here, the enzyme is a large single domain

dimeric protein with a three layer (αβα)-sandwich.[50] The active site is like for protease a catalytic triad consisting of Ser168, Asp223 and His280. In chapter 2.2.1. the mechanism of a catalytic triad is described in detail.[51] A specificity is that the catalytic triad is shielded by loops and helices which form a lid.[49] The lid is an α-loop of amphipathic character and thus the enzyme is only active at water/oil boundaries. In hydrophilic solutions the hydrophobic part of the lid faces the active site and the hydrophilic parts the solvent. As a result, the lid is closed and opens in the presence of hydrophobic solutions or interfaces.[52]

2.3. Overview of Enzyme Stabilization Methods

It is well known that enzymes lose their activity in liquid laundry detergents due to the contact to denaturing compounds like surfactants or chelators. Further, extreme temperatures and pH values inactivate enzymes. Additionally, protease degrades other enzymes and itself – especially if unfolded. As a result, enzymes in purchased liquid products are unfolded, destabilized, (autolytic) degraded or chemically modified and almost completely without the desired performance.[4, 53-55] However, the enzyme stability is not only an issue in detergent applications but also in other industrial usages. These may include animal nutrition, food processing, pharmaceuticals and biocatalysis.[56] The process conditions are often incompatible with a stable enzyme due to extreme pH values and temperatures or the presence of organic solvents.[57] As a consequence, the enzyme is denatured, so as the amino acid residues in the active site are too wide apart in order to perform and the enzyme loses its function.[58] Accordingly, it is necessary to formulate enzymes to ensure a maintaining of the enzyme function and performance.[59-60]

Today, a multiplicity of different strategies to increase the stability of enzymes are described in literature due to an increasing number of enzyme applications.[58] An overview of the different strategies for enzyme stabilization is shown in figure 2-6 and the strategies are described in the following sections.

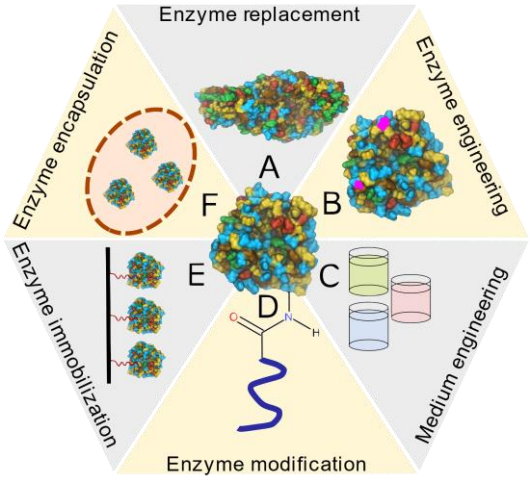


Figure 2-6: Illustration of different strategies for enzyme stabilization. A: enzyme replacement, B: enzyme engineering, C: medium engineering, D: enzyme modification, E: enzyme immobilization, F: enzyme encapsulation.

Enzyme Replacement

One obvious method to end up with a stable and active enzyme in an industrial process under harsh conditions is to replace the mesophilic enzyme (figure 2-6 A). There are enzymes in extremophile organisms that survive under conditions of extreme environments such as inter alia high temperature (up to 130 °C), high salt concentrations and extreme pH values (0-12).[61] Enzymes in such

microorganism are adapted optimally to the conditions, perform as a biocatalyst and could be the next generation for industrial biotechnology.[61-63] For instance, TOPLAK ET AL. identified a gene that encodes a serine protease in the thermophilic bacterium *Coprothermobacter proteolyticus*. The enzyme that results of the gene expressed in *Escherichia coli* is called proteolysin and shows enzyme activity up to 80 °C over a broad pH range. In addition, the thermophilic enzyme proteolysin shows a higher resistance to surfactants (10% sodium dodecyl sulfate, SDS) and organic solvents (ethanol and dimethyl sulfoxide, DMSO) compared with the mesophilic counterpart subtilisin A. Based on this results proteolysin is of great interest for the detergent industry.[64-65]

Enzyme Engineering

Instead of exchanging the whole enzyme another option is to change parts of the primary structure of an enzyme to enable a fitting to the present process specifications (figure 2-6 B)[66] Protein engineering got started in the 1980s[67-68] and can be broken down into two methods: rational protein design and directed evolution. Also Frances H. Arnold, a pioneer in the field of directed evolution, and honored by the Nobel Prize for Chemistry in 2018 has to be mentioned in this context.[69] Rational protein design means that being based on structures and sequences of proteins known as stable, new variants of the protein of interest are created through site-directed mutagenesis.[70] In contrast to rational protein design, for directed evolution an extensive knowledge of the protein structure is not required. In this case random mutagenesis followed by a selection of mutants with the desired enzymatic functions is implemented in a high-throughput.[71] With the objective of an organic solvent-stable lipase (*Candida antarctica*) PARK ET AL. used rational design based on findings from solvent-enzyme interactions. By targeted mutation of amino acids from the surface half-live of lipase in organic solvents could be increased up to 1.5 fold.[72]

Medium Engineering

Interactions between an enzyme molecule and the surrounding solvent molecules are crucial for the structural stability of enzymes and relating thereto for storage stability. For customers it is important that an enzyme retains its activity until it is used. For this reason, an appropriate medium to ensure a certain amount of enzyme activity and stability has to be engineered (figure 2-6 C).[73-74] It is most popular to add an additive to the enzyme aqueous solution to enhance the storage stability, whereby most enzyme formulations sold are stabilized with additives. These may include substrates, polyols, sugars, salts and polymers.[58] The addition of an additive that participates in the enzymatic reaction – a substrate or ligand – does not necessarily lead to the desired enzyme stabilization.[75] This strategy is

discussed further in chapter 2.4. Polyols and sugars added to aqueous enzyme solutions increase the hydrophobic interactions among non-polar amino acid residues. This leads to an enhanced thermal stability due to a rigidification of the protein.[73] The cause for this stabilizing effect is not clarified, but it is estimated that those additives have an effect on the water activity. This is either because of water molecules replacement from the enzymes´ hydration shell or because of the formation of a stabilizing shell around the enzyme which enables a preferential hydration.[73, 76-77]

Appropriately, osmolytes can shift the chemical equilibrium of native and denatured state to the more compact state – mostly the native active state.[78-79] NASIRIPOURDORI ET AL. increased the thermal stability and resistance towards proteolytic degradation of savinase, primarily used in detergents, by the addition of the two osmolytes sorbitol and trehalose.[80] Furthermore, an increased ionic strength by addition of salt can enhance the enzyme stability. The effect of salts has been first described by FRANZ HOFMEISTER in 1888. In this lyotropic series anions and cations are ordered as follows:[81]

$$N(CH_3)_4^+ > NH_4^+ > K^+, Na^+ > Ma^{2+} > Ca^{2+} > Ba^{2+}$$

$$SO_4^{-2} > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

Experiments with lysozyme have shown that if the enzyme is negatively charged, chaotropes (ClO_4^- or SCN^-) provide the unfolding and salting into solution whereas kosmotropes (SO_4^{-2}) support the stabilization of the native state and induce a salting-out effect. A positive charge of the enzyme results in an inverse Hofmeister series.[82-83] Not only small molecules can increase the stability of enzymes in an aqueous medium, but also polymers like polyethylene glycol (PEG) show a stabilizing effect due to an exclusion of enzyme from solvent parts and preventing denaturing effects.[58]

An elegant way of enzyme stabilization is the engineering of media that includes stabilizing molecules. In this way the enzyme storage stability can be increased and the enzyme itself is disposable in solution. An impairment of performance is not to be expected. However, stabilizing additives can interfere with final use reaction system. Regarding laundry applications the addition of Ca^{2+} might increase the stability of the enzymes but the cation interacts with anionic surfactants as described in chapter 2.1.2. In addition, for some additives high concentrations are required for a stabilizing effect. High concentrations can be difficult to be realized in the application or in the reaction systems and may be economically unviable.[58] Identification and design of enzyme specific stabilizing additives is quite difficult due to the complex structure and enzyme folding.

Enzyme Modification

Chemical modification of enzymes offers an alternative to enzyme engineering to achieve an enzyme tailored for an industrial process. The introduction of diverse groups to an enzyme via the amino acid sidechains is possible.[84] In the past, hydrophobic/hydrophilic groups have been introduced,[85] amino

acids have been phosphorylated or glycosylated[86] or crosslinkers like glutaraldehyde have been added to enzymes to enhance their stability.[87] It is a widespread strategy to link polymers covalently to enzymes.

Chemical modification of an enzyme is always associated with the risk of destroying the enzyme conformation and reducing its activity. Nevertheless, the modification of an enzyme leads to changes of the physical and chemicals properties like electrostatic interactions, hydrophobicity or hydrodynamic volume.[88-89] Modifications are often unspecific so it is unknown which amino acid residue is affected by the conditions used. Consequently, the performance of an enzyme can be decreased. Attachment of a polymer can result in enhanced molecular rigidity, which can lead to a thermal stabilizing effect.[58] The enzymes-polymer conjugates obtained are discussed in detail in chapter 2.5.

Enzyme Immobilization

Immobilization of an enzyme to a solid carrier is a common strategy that is especially attractive for applications in the biocatalysis. In that way the benefits of a heterogeneous catalysis can be enjoyed. In general, the operation control of the process and product separation without enzyme contamination are facilitated. Additionally, the enzyme usually retains its catalytic activity with an increased stability and can be reused for multiple cycles.[90-92] Enzyme immobilization to carriers like an inorganic polymer, a biopolymer or synthetic resin can be either via adsorption, covalent binding or entrapment.[93] Linkage between enzyme and carrier can occur via a single or a multipoint attachment. The latter is particular suitable in terms of an increased thermal stability.[94] Similar to the attachment of a polymer, enzyme immobilization to a rigid carrier can enhance molecular rigidity resulting in a thermal stabilizing effect.[58] Many positive reports from enzyme immobilization describe an increase in enzyme activity compared with the free native enzyme.[95-96] Observed activity loss can be attributed to enzyme denaturation or a hindered mass transfer due to the solid carriers.[97] In the latter case, the enzyme can be accidental linked to the carrier that the active site is blocked and not accessable for substrate. Unspecific enzyme adsorption takes this risk particularly.

One example for a successful enzyme immobilization is SINGH ET AL. who linked β-1,4-glucosidase from *Agaricus arvensis* covalently onto functionalized silicon dioxide nanoparticles. Thereby the enzyme shows an enhanced stability as well as a higher specific activity.[98] Entrapment is the inclusion of an enzyme in a polymer network that is synthesized in the presence of the enzyme.[99] It is widespread to use mesoporous silicates,[100-101] which are discussed in chapter 2.6 for enzyme entrapment. Another option to entrap enzymes is the use of metal-organic frameworks – as explained in chapter 2.7.

Enzyme Encapsulation

Enzymes can be encapsulated in a polymer network, a silica sol-gel or a microcapsule. The encapsulation in silica matrices is one of the most studied systems and stands out as an inexpensive, fast synthesis under mild conditions (further details in chapter 2.6).[102] With a water-in-oil microencapsulation nanoparticles with an undefined number of encapsulated enzymes are obtained.[103] Defined single enzyme nanoparticles (SENs) are received via a combination of enzyme modification and encapsulation. The enzyme is modified by an acryloylation to introduce polymerizable acrylic groups on the surface, followed by an *in situ* crosslinking polymerization.[104] In this way BELOQUI ET AL. encapsulated horseradish peroxidase (HRP) and demonstrated an up to 4-fold higher relative enzyme activity in organic solvents compared to the free enzyme.[105] Further encapsulation systems are microgels[106] or liposomes.[107] Encapsulation of enzymes behaves in a manner like immobilization. A further question is how to initiate the release of a successfully encapsulated enzyme. Encapsulation is not sufficient; the enzyme has to be released at the right time and as quick and complete as possible. The use of a temperature-responsive polymer, like Poly(*N*-isopropylacrylamide) (PNIPAm), is one possible very popular trigger for medical applications due to its biomedical compatible lower critical solution temperature (LCST) of 32 °C.[108] However, it is not suitable for laundry applications by reason of the varying storage temperature. In that case the immediately inflowing water can be utilized for an osmotic pressure release.

All six strategies mentioned have their own strengths and weaknesses and the optimal solution for an application depends on the enzyme as wells as the underlying process. When examining the distribution of publications on each strategy in the year 2017 (figure 2-7), it is noteworthy that by far most publications are on "enzyme engineering". Around half of publications with enzyme modification (keywords: "enzyme modification" and "enzyme conjugation"). Next with large distance comes "enzyme immobilization", followed by "enzyme encapsulation" and the least publications are on medium engineering (keywords: "enzyme medium engineering", "enzyme salt Hofmeister" and "enzyme salt stability") and enzyme replacement (keywords: "extremophile enzyme" and "thermophile enzyme"). It should be noted that not all publications dealing with one strategy are captured by the keyword search. In particular the topics medium engineering and enzyme replacement are undervalued. The corresponding pie chart is in figure 2-7.

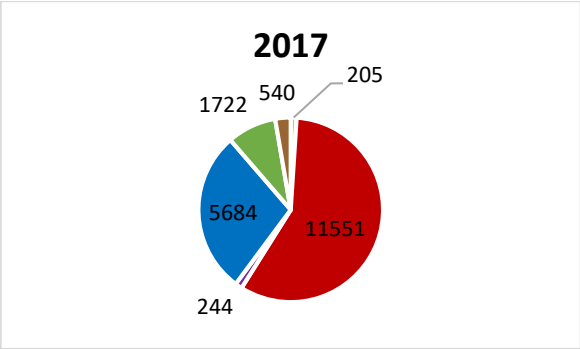


Figure 2-7: Distribution of publications 2017 (Web of Science) on enzyme replacement, enzyme engineering, medium engineering, enzyme modification, enzyme immobilization and enzyme encapsulation.

Often a strict distinction between the strategies is unfeasible. Rational protein design for instance can refer to an extremophilic enzyme to enhance the properties of a mesophilic enzyme. Both strategies are used widely from biologists or biochemists at the beginning of the development of a new enzyme. On the contrary, medium engineering, enzyme modification, immobilization and encapsulation are strategies based on the final enzyme engineered.

In addition to the scientific issues, industrial frameworks must be considered. The strategy used for enzyme stabilization must be economical viable and the technology has to be feasible. Environmental belongings must be kept in mind as well. The present work is focused on four different methods to increase the stability of enzymes in a liquid detergent formulation versatile within an industrial context. These strategies are small molecules (chapter 2.4), enzyme-polymer conjugates (chapter 2.5), mesoporous silica systems (chapter 2.6) and metal-organic frameworks (chapter 2.7). In figure 2-8 the four methods are illustrated. Those methods fall into the categories medium exchange, enzyme modification and immobilization or rather encapsulation and are described in detail in the following sections.

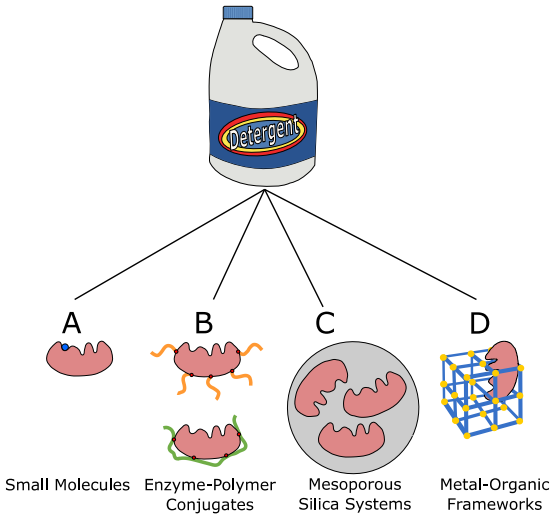


Figure 2-8: Overview of the four enzyme stabilization strategies used in the present thesis. A: small molecules, B: enzyme-polymer conjugates, C: mesoporous silica systems, D: metal-organic frameworks.

2.4. Small Molecules

This chapter of small molecules states the first strategy used to stabilize enzymes in a liquid detergent formulation and belongs to the technique of “medium engineering” mentioned in chapter 2.3. In the following subsections the influence of small molecules on enzymes in general is described (chapter 2.4.1.), small molecules already in use for laundry applications are mentioned (chapter 2.4.2.), the open research question is formulated (chapter 2.4.3.) and finally, the concept of the present work is explained (chapter 2.4.4.).

2.4.1. Small Molecules and Enzymes

Small molecules can influence the activity and stability of enzymes to the positive as well as to the negative side. The presence of substrates, co-factors and inhibitors effects the stability of enzymes.[109] Specially known are enzyme inhibitors which are molecules that interact with an enzyme and decrease its activity. This mode of action is base of many drug molecules and therefore of pharmacological interest. Regarding the mechanism it is possible to differentiate between reversible inhibition and irreversible inactivation. The first case is further divided into competitive, uncompetitive and non-competitive inhibition.[40] The types of inhibition and inactivation are shown in figure 2-9.

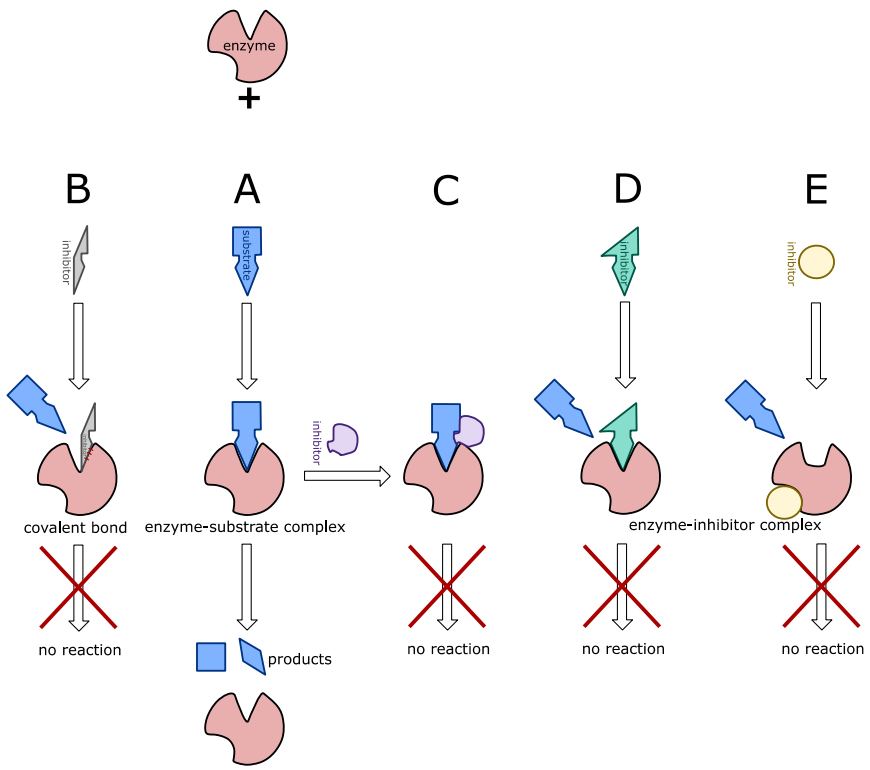


Figure 2-9: Types of enzyme inhibition. A: normal enzyme reaction; B: irreversible inactivation; C: reversible uncompetitive inhibition; D: reversible competitive inhibition; E: reversible non-competitive inhibition.

In a normal enzyme reaction (figure 2-9 A) substrate and the active site of an enzyme interact and form an enzyme-substrate complex. As a consequence of the interaction, chemical bonds within the substrate are cleaved and the substrate decomposes into products or chemical bonds are formed and a product is built up. The inactivation of enzymes due to a modification by covalent binding of an inhibitor (figure 2-9 B), is referred as irreversible inhibition. A dissociation of inhibitor and enzyme is not possible, and the enzyme remains inactive. If the interactions between enzyme and inhibitor are non-covalent so that a dissociation is possible, a reversible inhibition exists. In most cases of inhibition, the inhibitor can bind to the enzyme´s active site and competes with the substrate for binding accordingly (figure 2-9 D). Due to the block of the active site, turnover of the substrate is reduced, but can be recovered if the concentration of the real substrate is enhanced. Within a non-competitive inhibition, the inhibitor binds to a site other than the active site (figure 2-9 E). Binding of the inhibitor to the so called "allosteric site" results in a conformational change of the active site. The formed enzyme–inhibitor complex prevents the interaction between enzyme and substrate and cannot repealed by an increased addition of substrate. In the last case of reversible inhibition, the inhibitor can only interact with the larger enzyme-substrate complex and not with the enzyme alone (figure 2-9 C). Binding of the inhibitor to the complex is called uncompetitive inhibition and leads to a stop of substrate decomposition.[110]

Besides enzyme inhibition, it is known that low molecular weight substances can influence enzyme stability in aqueous solutions. The effect can be either stabilizing or destabilizing. For instance, guanidine hydrochloride and urea unfold and denature proteins. Whereas polyols like sucrose or glucose and salts such as ammonium sulfate exert a stabilizing effect.[75] In general, the stabilizers can be divided into osmolytes and ionic stabilizers.[111] Osmolytes are for example polyols such as glycerol and dipolar molecules like trimethylamine *N*-oxide. They are almost uncharged and influence the solvent viscosity as well as the surface tension. In addition, they stabilize a hydration shells and avoid protein aggregation.[111] Up to concentrations of at least 10 to 40 wt-%, osmolytes have only little effects on enzyme activity and stability.[112] Ionic stabilizers like salts (e.g. phosphates or quaternary amines) can shield surface charges for stabilization at low concentrations. They tend to initiate protein precipitation at high concentrations by competing with water molecules.[111]

The presence of ligands has various effects on enzyme stability. A ligand participates in enzymatic reactions and binding can lead to a stabilization, destabilization or has no effect.[75] CIMMPERMAN ET AL. predicate the effect observed on the preferred binding of the ligand. Accordingly, a destabilization can be observed if the ligand binds primarily to the unfolded state of the enzyme.[113]

2.4.2. Small Molecules for Enzyme Stabilization in Liquid Detergents

The use of small molecules for enzyme stabilization in liquid detergents is attractive due to the waiver of enzyme encapsulation and subsequently, circumventing a complicated enzyme release. Additionally, small molecules are often easy to formulate and bear a small risk of soiling textiles. In general, the amount of enzyme stabilizers within household detergents should not exceed 8 wt-%.[114] Together with the first liquid detergents entering the market, enzyme-stabilization systems in form of small molecules were added to the solutions. First stabilizers have been mixtures of polyfunctional amino compounds such as triethanolamine or polyols like sorbitol in combination with boric acid derivatives or borax.[115] Alternatively, water-soluble formats[116] and calcium ions (for example 0.08 wt-% calcium chloride[114]) are added to liquid detergents.[117] The effectiveness of sodium formate depends on the pH value and is optimal below eight. Detergent formulations possess a pH above eight which limits the stabilizing effect of that formate. Addition of high concentrations of calcium ions results in precipitation of surfactants and is therefore unfavorable. Propylene glycol and glycerine are popular additives despite their high concentrations required for enzyme stabilizing effects.[118]

The additives mentioned above interact mostly unspecifically with all enzymes and form the basis of enzyme preformulations. Small molecules for specific enzyme stabilization in liquid detergents are designed mostly for proteases. Protease – as a catalyst of peptide bond proteolysis – must be inhibited during storage to protect the other enzymes and itself from degradation. Many protease inhibitors are known, but only very few are suitable for laundry applications. For example, an irreversible enzyme inactivation by a serine protease inhibitor like phenylmethylsulphonyl fluoride (PMSF) would not be appropriate. The proteolytic activity should be restricted only temporarily and lifted during the washing process. This is not the case for strong covalently acting inhibitors. A reversible competitive inhibition of protease is one option to control the proteolytic activity. The inhibitor stabilizes the enzymes in the concentrated detergent during storage and due to the dilution in the washing process, the inhibition is abrogated and the protease becomes active.[15] Boric acid is widely used and acts as a competitive inhibitor for serine proteases and therefore has been added to liquid detergents. Crystal structure and NMR experiments indicates that boric acid forms a hydrogen bond to Asp32 and an ion pair with the His64 in the catalytic triad.[119] However, boric acid has disadvantages: firstly, boron is reprotoxic.[120] And secondly, boric acid complexes with the often-used builder citric acid and loses its inhibition properties.[121]

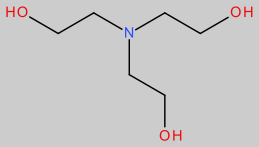
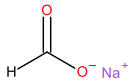
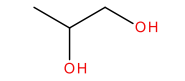
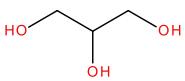
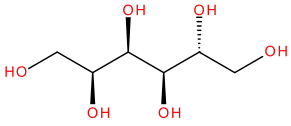
For this reason, Novozymes AS screened for more efficient alternatives and identified 4-formylphenylboronic acid (4-FPBA).[122] Since 1995 4-formylphenylboronic acid (4-FPBA) is added with an amount less than 0.08 wt-% to liquid laundry solutions to reduce the proteolytic activity and increase the storage stability of the enzymes.[122] 4-FPBA represents a 100 times more potent inhibitor than boric acid, although the presence of boron is still a health hazard. In addition, the inhibition effect

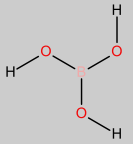
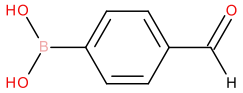
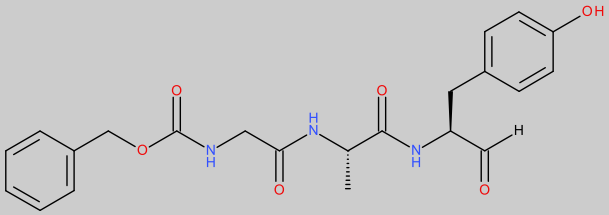
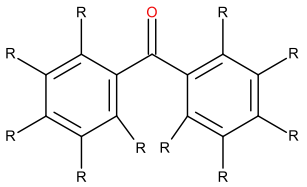
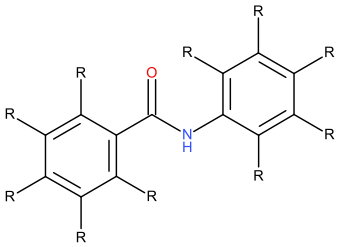
is reduced in presence of builders. Nevertheless, the combination of polyols and boronic acid derivatives is the most commonly used stabilization technique for liquid detergents.[120]

Recent developments shift their attention from boronic acids and focus on healthier alternatives as second generation inhibitors like peptide aldehydes. Peptide aldehydes are oligopeptides consisting of two to five amino acids having a reduced *C*-terminus. The reversible inhibitor forms a hemiacetal with the protease active site.[119] Peptide aldehydes are effective in low concentrations and less susceptible to chelating agents. From the seller´s point of view, peptide aldehydes are more expensive than boronic acids and sensitive to oxidation.[123] Further alternatives for protease inhibition are benzophenone and benzanilide derivatives containing carboxyl groups[124] as well as phosphoric acid diesters.[125]

Table 2-2 summarizes the small molecules used as enzyme stabilizers in liquid detergents and points out the disadvantages of each system.

Table 2-2: Tabular summary of small molecules used as enzyme stabilizers in liquid detergents, their target enzymes and disadvantages and structures.

Small molecule	Targeted enzyme	Disadvantage	Structure
Polyfunctional amino compounds	Protease α-amylase lipase	High concentrations	 Triethanolamine
Sodium format	Protease α-amylase lipase	pH value < 8	
Calcium chloride	Protease α-amylase	Precipitation of surfactants	$CaCl_2$
Propylene glycol **Glycerin** **Sorbitol**	Protease α-amylase lipase	High concentrations	  

Boric Acid	Protease (α-amylase) (lipase)	Reprotoxic	
4-FPBA	Protease	Reprotoxic, builders reduce effect	
Peptide Aldehyde	Protease	Expensive Sensitive to oxidation	 Z-Gly-Ala-Tyr-CHO
Benzophenone Benzanilide	Protease	Poorly soluble in water	  R: hydrogen, halogen, carboxy, methyl, ethyl, hydroxyl, hydroxymethyl, amino group

2.4.3. Small Molecules in Detergent Applications: Open Research Questions

It should be mentioned, that all specific small molecules listed in table 2-2 target protease for inhibition. The prevention of proteolysis serves to enhance the stability of protease and is accompanied with an increased stability of all detergent enzymes. However, apart from the proteolysis problem, denaturing surfactants and builders are present as well in a liquid detergent formulation. Additionally, possible temperature fluctuations can lead to an enzyme denaturing and a combined loss of function. For this reason, other enzymes than protease – α-amylase and lipase for instance – need protection and stabilization systems, too. As far as currently known, there are no stabilizers addressing specific lipase on the detergent market. To fill this gap, a selection of small molecules targeting lipase has been identified and tested in the present thesis. Thereby, the question whether it is possible to

identify a small molecule targeting lipase specifically to enhance lipase stability without a negative effect on the other enzymes is pursued.

2.4.4. Small Molecules in Detergent Applications: Own Strategy

Acetylcholinesterase (AChE) is like lipase part of hydrolase enzymes and catalyzes the cleavage of the neurotransmitter acetylcholine into choline and acetic acid (figure 2-10).[126] Both enzymes possess a catalytic triad in their active site and show in the family tree of hydrolases a relatively close relationship.[126]

With AChE as a starting point, the concept is that the detergent lipase recognizes the substrate (acetylcholine) of the related AChE. Due to the relation, acetylcholine should bind to the active site of lipase. Further investigations in chapter 5.2.3 showing a pH drop indicate that the ester bonds are cleaved by lipase.

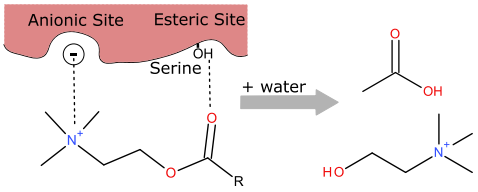


Figure 2-10: Hydrolysis of acetylcholine to acetic acid and choline.

Besides acetylcholine, three related structures are tested for lipase stabilization: citric acid choline ester, triethyl citrate and acetyl triethyl citrate. The compounds are displayed in figure 2-11. Acetylcholine (figure 2-11 A) bears one positive charge. Citric acid esterified with approximately two choline chlorides has two positive charges (figure 2-11 B). Based on citric acid choline ester, citric acid three times esterified with acetic acid (figure 2-11 C). Finally, the free hydroxy group of triethyl citrate is esterified as well resulting in the very hydrophobic acetyl triethyl citrate (figure 2-11 D).

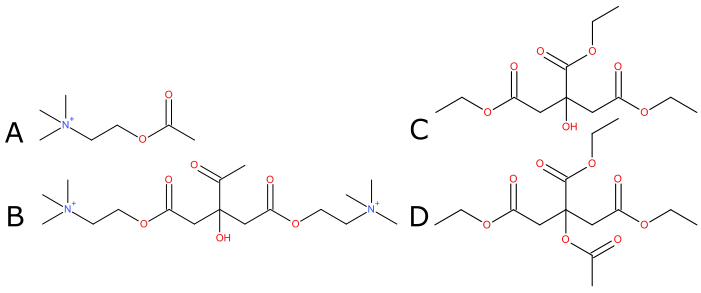


Figure 2-11: Small molecules tested for lipase stabilization. A: acetylcholine, B: citric acid choline ester, C: triethyl citrate, D: acetyl triethyl citrate.

2.5. Enzyme-Polymer Conjugates

The second strategy – enzyme-polymer conjugates – described here belongs to the strategy "enzyme modification" (chapter 2.3., figure 2-6 D). Firstly, a general overview of enzyme-polymer conjugates is given (chapter 2.5.1.). Secondly, the synthesis behind and recent developments in the area of enzyme-polymer conjugates are mentioned (chapter 2.5.2.). Afterwards, the open research questions are asked (chapter 2.5.3.) and finally, the strategy used is stated (chapter 2.5.4.).

2.5.1. Enzyme-Polymer Conjugates: General Introduction

Enzyme-polymer conjugates are enzymes that are modified due to a covalent attachment of a polymer. It finds its application mostly for pharmaceutical purposes,[127-128] thereby the poor solubility and stability of proteins *in vivo* should be increased.[129] Besides, the retention time in the organism should be extended and the potential for an undesired immunogenic effect reduced.[130-131] However, polymers used for enzyme conjugation should be inert, water-soluble and biocompatible. Known from literature is a variety of polymers like PEG,[132-133] hydroxyethyl starch (HES),[134] dextran[135] or polyvinylpyrrolidone (PVP).[136] The subsequent two paragraphs review PEG and polysaccharides as polymers for enzyme conjugation. These are the most commonly used polymers for enzyme-polymer conjugation and their properties make an application in detergents conceivable.

First conjugates with PEG and a protein have been synthesized in the 1970s by DAVIES and ABUCHOWSKI.[137-138] Posterior, the term PEGylation which describes the covalent attachment of PEG to a protein has been introduced.[139] Generated benefits due to PEGylation – besides pharmaceutical purposes – are an increased stability against proteolytic degradation[140-141] and the possibility to dissolve proteins in organic solvents.[142] In general, unilateral methylated PEG is used for conjugation in order to prevent crosslinking and aggregation which could occur with PEG diol.[143] PEG itself is the most commonly used polymer for conjugation and is approved by the Food and Drug Administration (FDA).[140-141, 143] PEG has two beneficial properties for the formation of enzyme conjugates: the polymer chain is highly flexible and the backbone is highly hydrated owing to the coordination of about six to seven water molecules per monomer unit.[144] Consequently, PEG has a good solubility in water and in many organic solvents. However, PEG is not biodegradable so the polymer accumulates in the cytoplasm of kidney cells.[145] Additionally, with high degrees of PEGylation anti-PEG antibodies can be formed *in vivo*.[146-148]

Polysaccharides are also widely used for enzyme-polymer conjugates and they are characterized by a defined structure, a high availability and a good water solubility as well as biocompatibility and -degradability.[145] In the style of PEGylation a conjugation with HES is called HESylation.[134] It has to be taken into account that about 50% of all enzymes are glycosylated naturally for an improved

stability.[149] The natural glycosylation can take place at serine and threonine (O-linked glycosylation) or asparagine (N-linked glycosylation). In contrast, artificial glycosylation is realized with lysine in most cases.[150]

2.5.2. Enzyme-Polymer Conjugates: Synthesis and Recent Developments

In general, the conjugation reaction is influenced by the enzyme to polymer ratio, reaction time, temperature and pH value.[139] The conjugation itself proceeds between an activated functional group on the part of the polymer and reactive and accessible amino acid residues by the enzyme. Hydroxy groups, available at polymers like polysaccharides or PEG show a low reactivity and must be converted into reactive electrophilic groups to react with the nucleophilic amino acid residues under physiological conditions. The reaction temperature should be between 4 °C and room temperature and the reaction should take place in an aqueous medium in a pH range of 4.5 to 9 so that the enzyme stays in its native conformation.[151-152] Thereby, the reactivity of amino acids for the synthesis of enzyme-polymer conjugates depends on the particular pK_a value and the exposure of the amino groups on the enzyme surface.

With respect to the reactivity following grading exists: thiol > α-amine > ε-amine > carboxyl > hydroxyl.[153] Due to its nucleophilic thiol group cysteine is the most reactive amino acid. However, this amino acid is relatively rare in the sequence of enzymes, often located inside an enzyme or blocked via disulfide bonds.[154] There are experimental approaches to obtain free thiol groups. Disulfide bonds can be cleaved by the addition of reagents like dithiothreitol, but this can be combined with a loss of the 3D structure and the enzymes´ activity.[155] A further possibility is the conversion of primary amines to thiols with the use of 2-iminothiolane (Traut´s reagent).[156] It is also possible to modify an enzyme recombinantly to integrate additional cysteines in the sequence.[156-157] Thiol groups can react with electrophilic groups – like vinylsulfone or maleimide – under slightly acidic to basic conditions (pH 6 to 7). Under these conditions no competing reaction with the amino groups will occur.[158-159]

Most commonly used residues are the amino groups of enzymes. They are located at the side chain of lysine as well as at the *N*-terminus. A large number of possible electrophilic polymer groups is known in literature.[153] A distinction has been made between acylating and alkylating reactions. Via an acylating reaction with *N*-hydroxysuccinimide (NHS) as an active ester, amides can be synthesized in a fast reaction under physiological conditions (figure 2-12 A).[153, 160]

An example for an alkylation is the reductive amination with an aldehyde (ald). The first step of this reaction is the reversible nucleophilic addition and the formation of an imine (Schiff base). To end up with a stable covalent linkage the imine can be reduced further to a secondary amine (figure 2-12 B). Sodium cyanoborohydride is a suitable reducing agent that attacks the imine selectively.[153, 161] The pH

value has an influence on the selectivity of this reaction. If the pH is adjusted to 5 to 6, the conjugation takes place at the *N*-terminus due to a lower pK_a value compared to the lysine residues. In this way it is possible to conjugate one polymer per enzyme.[156, 162-164] In figure 2-12 the conjugation between amino groups and NHS active ester as well as aldehyde is shown.

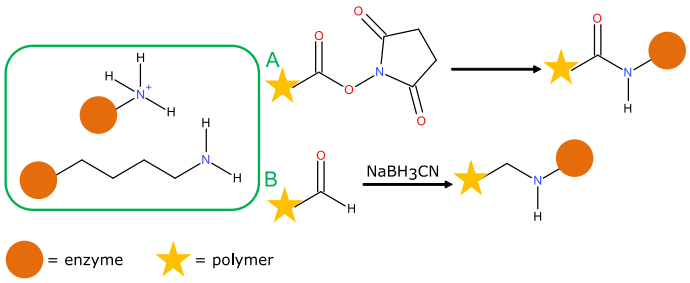


Figure 2-12: Conjugation reaction between amino groups from the enzyme (N-terminus and lysine residues) and NHS active ester (A) and aldehyde (B).

Recent Developments in Enzyme-Polymer Conjugates

The strategy of enzyme-polymer conjugates arises in the 1970s with the covalent attachment of PEG-1900 and PEG-5000 to bovine liver catalase by ABUCHOWSKI ET AL. Coupling agent has been 2,4,6-trichloro-s-triazine and about 40% of the amino groups of the enzyme have been modified. Despite the conjugation the catalase used retains its enzymatic activity almost completely and shows additionally an increased stability against digesting enzymes.[137] Years later, several PEGylated proteins are used in clinical practice and the possibilities for the formation of enzyme-polymer conjugates extended into infinite space.[165] The conjugation site and the number of polymer chains attached per enzyme unit is essential for enzyme-polymer conjugation regarding stability and activity of the conjugates.[166] Thereby, the technique evolves from the random conjugation of amino groups to a highly refined technology. Random coupling can lead to problems in the reproducibility from batch to batch and to a more complicated characterization. Additionally, the active site of an enzyme is more vulnerable to sterically hindering in a random conjugation.[167] Current research is mainly focused on a site-selective conjugation to obtain one homogenous isomer and uses novel approaches like genetic engineering to insert unnatural amino acids for biorthogonal click chemistry or enzymatic PEGylation.[166] A second enzyme is able to site selectively catalyze the reaction between a polymer and a specific amino acid of the enzyme of interest. Physiological reaction conditions can be used, and high yields are common. Transglutaminases for instance can transfer in a cross-linking reaction the acyl moiety of glutamine residues to linear primary amines, like amino-PEG.[168] Glutamine residues used as substrate have to be highly flexible and at the surface of an enzyme, which results in a selectivity and

at best in homogeneous monoPEGylated enzymes.[169] The choice of solvent can increase the selectivity[170] as well as an immobilization of transglutaminase. GRIGOLETTO ET AL. immobilized transglutaminase on an inert polysaccharide resin. The PEGylation of α-lactoalbumin is more selective and results in the formation of one monoconjugated derivative. In addition, the immobilization simplifies the purification and removal of transglutaminase.[171]

In consequence of the limitations and disadvantages of PEG, like the above mentioned kidney accumulation and the formation of antibodies, alternative polymers are used for enzyme conjugation nowadays, which include for example HES,[172] hyaluronic acid,[173] dextrin[174] and polyoxazoline.[175] KONIECZNY ET AL. POXylated inter alia lysozyme with different polyoxazoline-derivatives and enable a solubility of the enzyme in methanol, ethanol, chloroform, toluene and tetrahydrofurane (THF).[176]

Another trend is towards conjugation with stimuli-responsive polymers that respond to a change of temperature for instance. An enzyme conjugated to a temperature-responsive polymer can benefit from a protective layer if the temperature is raised and the polymer precipitates consequently.[167] SHAKYA ET AL. synthesized conjugates of bovine liver catalase and PNIPAm. The conjugates obtained show a temperature-responsive behavior with a decreased LCST at 26 instead of 32 °C. Thermal and storage stability of catalase-PNIPAm conjugate are improved compared to native catalase.[177]

Within the recent developments the needs of the existing problem and present application must be considered. For instance, in the case of a detergent application the use of PNIPAm has no positive impact on the enzyme formulation. In the following chapter open research questions are portrayed.

2.5.3. Enzyme-Polymer Conjugates in Detergent Applications: Open Research Questions

Chemical modification of proteins with natural or synthetic macromolecules is well studied and has become an established technology to improve the stability of enzymes. Despite the large number of working groups dealing with the topic of enzyme conjugation, research examples considering real detergent application conditions are very limited. The increased enzyme stabilization against individual surfactants like SDS due to conjugation for instance is described by GAERTNER ET AL. who PEGylated trypsin and measure the relative enzyme activity after 30 minutes incubation.[178] SCHROEDER ET AL. studied the stability of PEGylated protease for a short-term of two hours and focus more on the protection of the textile which could be damaged by the enzyme.[179] In contrast, in this work the enzyme stability over a long-term period of four weeks within a complete liquid detergent formulation is of interest. In addition, a combination of two enzymes (protease and conjugated lipase) is investigated, because of the sensitivity of lipase towards proteolysis. It will be examined whether the conjugation of enzymes with polymers can increase the stability of the enzyme against a detergent formulation as well as against proteolysis. In this connection it will be studied if the detergent enzymes

can be conjugated successfully and additionally, two strategies – PEGylation and glycosylation – will be compared.

2.5.4. Enzyme-Polymer Conjugates in Detergent Applications: Own Strategy

For this analysis four different polymers are tested. On one side two methylated PEG (mPEG) with a single end functionalization – aldehyde and NHS active ester – are used (figure 2-13). Both chains possess a molecular weight of 5000 Da and are reactive towards amino groups. On the other side two polysaccharides – CMC and maltodextrin – are used for enzyme glycosylation (figure 2-13 B). Therefore, the polysaccharides are oxidized partially to introduce amino reactive aldehyde groups in their side chain. In contrast to the functionalized mPEGs, more than one reactive aldehyde per polymer chain is present in case of the oxidized polysaccharides. However, the option of a multipoint attachment between polymer and enzyme makes the two polysaccharides attractive due to a literature known increase in thermal stability.[94] The conjugation conditions chosen for all four polymers target as much amino groups as possible. According to the literature the thermal stability of an enzyme increases with rising number of polymer chains attached until a saturation is reached.[180] A present inhomogeneity of the enzyme-polymer conjugates obtained is not decisive for a laundry application and carries more weight for drug applications due to stringent requirements.

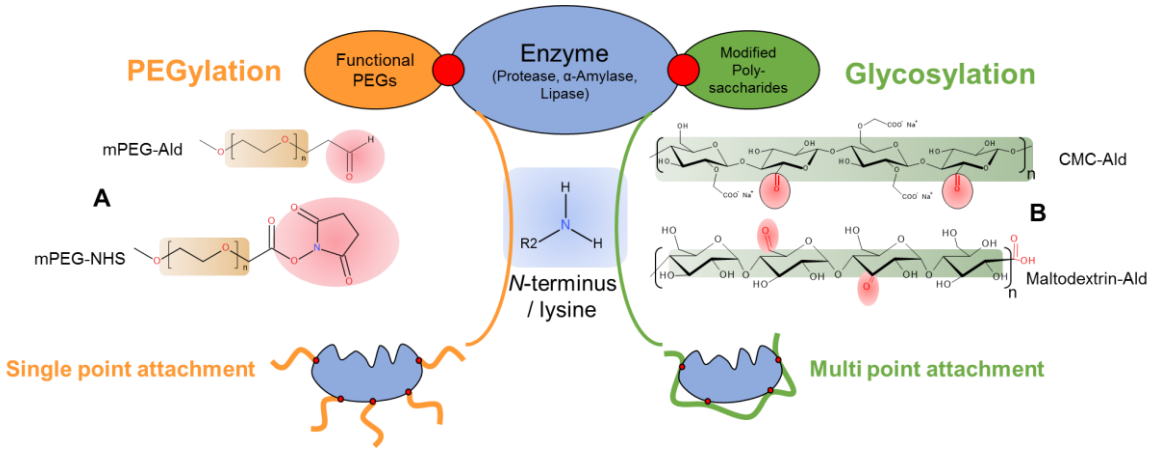


Figure 2-13: Formation of enzyme-polymer conjugates via two different strategies. A: PEGylation with mPEG-aldehyde and mPEG-NHS. B: Glycosylation with CMC-aldehyde and maltodextrin-aldehyde. Red cycles indicate the functional group reactive toward covalent coupling with enzymes´ amino group.

Expected Challenges

In the present work enzyme modification via random conjugation of amino groups is used to address the question if enzyme-polymer conjugation can increase the stability of enzymes in liquid detergent formulations. This kind of conjugation is accompanied by the following expected challenges:

- Low degree homogeneity → complex mixtures of conjugates enzymes (especially with the two polysaccharides)
- Reproducibility problems due to random conjugation
- Steric blocking of the enzyme active site
- Crosslinking and formation of aggregates using polysaccharide-aldehydes for conjugation

2.6. Mesoporous Silica Nanoparticles

In this chapter the third strategy used to stabilize enzymes in liquid detergents is illustrated: encapsulation in mesoporous silica nanoparticles. This strategy belongs to the technique of “enzyme encapsulation” (chapter 2.3., figure 2-6 F). The first of the following subsections contains a general introduction into the topic of mesoporous silica nanoparticles (chapter 2.6.1.). Afterwards, the topic is associated with enzymes (chapter 2.6.2.) and the open research question is formulated (chapter 2.6.3.). At the end, the strategy of the present work is stated and expected challenges are listed (chapter 2.6.4.).

2.6.1. Mesoporous Silica Nanoparticles: General Introduction

A mesoporous material is a porous inorganic solid with a pore diameter between 2 and 50 nm usually possessing a cubic or hexagonal structure.[100] Silica-based mesoporous nanoparticles (NP) are most studied, but other types such as alumina or titania have been used as well.[181] Generally, particle size, structural order, pore diameter, wall thickness and stability of NP can be varied and customized for the application.[182] Those characteristics make silica NP attractive as drug delivery system starting 2001 with ibuprofen.[183] Not only small molecules can be loaded into mesoporous materials, but also large molecules like enzymes can be encapsulated.[184]

Mesoporous materials are formed by an organic-inorganic self-assembly between an inorganic precursor and a surfactant (template). For the synthesis, the template is dissolved in an aqueous solution to form micelles. Then the precursor is added, hydrolyzed, condensed and polymerized to form an inorganic network around the self-assembled template. Afterwards, the material formed can be further modified by hydrothermally treatment to increase crosslinking and tune the pore size. Finally, the template is removed by extraction or calcination to obtain the mesoporous material.[100] The process is shown in figure 2-14.

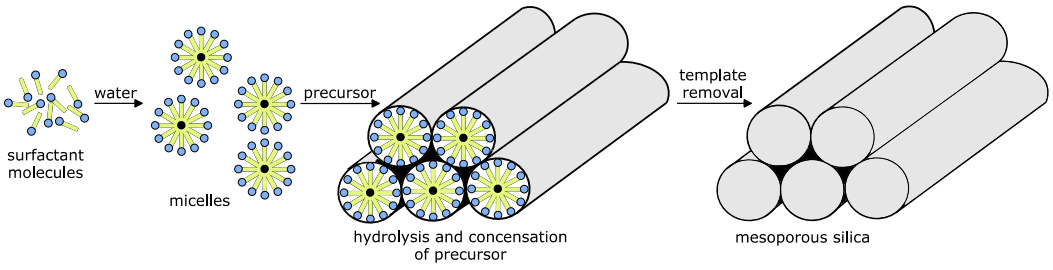


Figure 2-14: Representation of the formation of mesoporous silica materials.

Size and morphology of mesoporous silica NP depends on the hydrolysis rate and its synchronization with the condensation of the inorganic silica precursor. These processes can be varied by adjustment of pH and stirring rate as well as choice of template and solvent.[185-186] OZIN ET AL. observed that a slightly acidic pH results in the formation of spherical NP with a size of 1 to 10 µm.[187] Depending on the stirring rate long fibers (slow stirring) or a fine powder (fast stirring) can be formed.[188] From an analytical point of view, particle size can be determined using electron microscope or dynamic light scattering (DLS).[189] Varying the amount of silica precursor and template, the pore size of the resulting mesoporous silica NP can be adjusted.[185] Again the pH value during the synthesis influences the pore structure based on its influence on hydrolysis and condensation reaction rates which have to be synchronized with template assembly. At pH values between 10 and 12 hexagonal structures are formed, whereas at pH values above 12 a lamellar meso phase is produced.[190] In addition, the choice of surfactant used as template influences the pore size significantly.[191] As mentioned before, hydrothermal treatment can be used to tune the pore width further. A freshly prepared mesoporous material can be exposed to autogenic pressure at elevated temperatures, optional in the presence of additives. In that way the pore size can be increased without influencing the morphology of the material.[185, 191] Using transmission electron microscopy (TEM) and X-ray diffraction the pore size of mesoporous silica NP can be analyzed. Nitrogen or less faulty argon sorption can be used to determine the pore width.[191]
The surface of mesoporous silica NP can be functionalized by organic groups to control the absorption and release of drugs or proteins.[192] There are almost no limits regarding the functionalization of pore walls. For instance, VALLET-REGI ET AL. functionalized the pore of mesoporous silica NP with amino groups to facilitate the incorporation of a drug.[193]

2.6.2. Mesoporous Silica Nanoparticles and Enzymes

One opportunity to increase the stability of enzymes under nonphysiological conditions is the immobilization or encapsulation in mesoporous silica NP. Due to the advantages for enzyme applications especially in biocatalysis, the use of mesoporous materials have been explored extensively during the last years.[100] The properties of mesoporous silica NP, like the well-defined pore geometry or the narrow pore size distribution, make them suitable for the immobilization of many different sized enzymes.[194] Enzymes can be wrapped completely into a NP and in that way protected against a denaturing environment.[195] In addition, the dissociation of a multimeric enzyme into subunits can be prevented, enzymes keep together and stability is increased. Further advantages are the tunable pore size and the opportunity to modify the silica surface. Both enable a further control of enzyme stability and activity. Generally, the synthesis of mesoporous silica NP takes places at mild conditions with inexpensive chemicals and biocompatible products.[196]

The combination of enzymes and mesoporous silica NP can take place via four frequently used approaches,[196] that are shown in figure 2-15: physical adsorption (A), covalent binding (B), cross-linked enzymes aggregates (CLEAs; C) or one-pot synthesis (D).

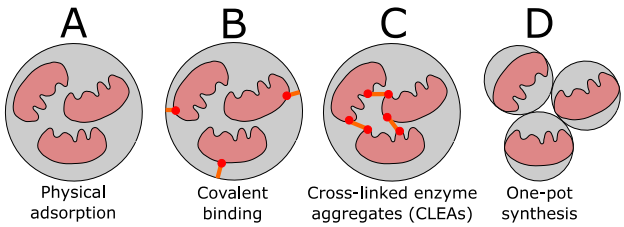


Figure 2-15: Different routes for immobilization and encapsulation of enzymes into mesoporous silica NP. A: physical adsorption, B: covalent binding, C: CLEAs, D: one-pot synthesis.

If the mesoporous silica material is synthesized firstly, route A to C (figure 2-15) are available for selection to load enzymes into the material with suitable pore structure. Using physical adsorption (figure 2-15, A) the interactions between the porous support and the enzyme are noncovalent and therefore mainly Van der Waals forces or electrostatic forces as well as hydrogen bonding and hydrophobic interactions are present.[194] Thereby, electrostatic interactions are the strongest, though, they depend on the pH value and the isoelectric point (pI) of the enzyme.[197] In general, enzymes with a pI below 7 are difficult to encapsulate due to the electrostatic repulsion between the own negative charge and the negatively charged silica surface. For such occasions silica materials can be replaced by titania or alumina, both with a considerable higher pK_a of five respective eight than silica with about two.[198] Adverse is the faster hydrolysis of titania and alumina precursors compared with silica precursors, resulting in difficulties regarding ordered structures.[199] Alternatively, the surface of the silica material can be modified to enable or maximize electrostatic interactions to enzymes. For instance, charged organic moieties like carboxylic, phosphoric acid, sulfonic acid or amine groups can be introduced.[200] However, enzymes immobilized by electrostatic interactions can be released from the mesoporous material by pH changes. To prevent leaching, the size of the pore entrance can be reduced after enzyme adsorption by introduction of bulky functional groups.[194] For instance, WANG ET AL. deposited a multilayered polyelectrolyte shell onto the enzyme-loaded spheres to prevent leaching and to enhance catalase stability.[201] Nevertheless, there is a risk that the enzyme is affected or denatured by the chemical modification reaction.

A second possibility is the covalent linking of enzymes to the NP by chemical bonding (figure 2-15, B). Using this strategy, the enzyme is fixed onto the support and leaching is prevented. Contrarily, an easy release of the enzyme is not possible anymore. Mesoporous silica materials are proven highly appropriate for covalent enzyme bonding. The silanol groups on the surface can be functionalized and

modified post synthesis (grafting). Alternatively, via co-condensation the direct synthesis and introduction of organic groups is possible.[194, 202] Comparable with the previously described enzyme-polymer conjugates (chapter 2.5.), the most conventional binding site in enzymes for covalent binding are the amino groups from lysines or – if available – thiol groups from cysteines. The surface of the mesoporous material can be functionalized with amino groups as well. Therefore, homobifunctional crosslinkers like glutaraldehyde (GA) or succinimido-3-maleimidopropanoate are used for the covalent bonding between silica NP and enzyme.[203] A direct reaction with the enzyme under mild conditions is possible if epoxy groups are introduced on the NP surface.[204] Further opportunities are the Cu(I)-catalyzed click reaction between an azide functionalized silica surface and alkyne modified enzyme[205] or the thiol-ene Michael addition between thiols and activated double bounds.[206]

In the place of binding the enzyme covalently to mesoporous silica NP, the biocatalysators can be cross-linked itself (figure 2-15 C). So called cross-linked enzyme aggregates (CLEAs) can be obtained by the addition of homobifunctional crosslinker like GA.[207] Using this strategy, enzymes are physically adsorbed in the pores before the crosslinker is added and CLEAs are formed. The formed aggregates are significantly larger (0.1 to 200 µm) than single enzymes and therefore entrapped in the pores. In this way leaching is prevented and substrates can still diffuse to the enzymes. On the other hand, crosslinking of enzymes can be combined with a loss of enzyme activity.[194]

The last strategy to entrap enzymes into mesoporous silica NP is one-pot synthesis (figure 2-15, D). All strategies described before have in common that the mesoporous material is synthesized in a first step and the enzyme is adsorbed into the pores in a second step. In a one-pot synthesis the mesoporous material is formed in presence of the enzyme around the enzyme. Accordingly, the enzyme is added to an SiO_2 precursor and the reaction conditions have to be adjusted to the enzymes´ needs to avoid a deactivation.[208]

Using mesoporous silica materials as support for enzyme immobilization or encapsulation have been investigated extensively during the last decades. Applications are biocatalysis, biosensing as well as drug delivery. All strategies described in this chapter are used for this purpose. Physical adsorption of the enzyme into a prior synthesized mesoporous material is despite to the leaching problem an often-chosen method for enzyme encapsulation (figure 2-15, A). For instance, KALANTARI ET AL. immobilized lipase into mesoporous silica nanoparticles further modified with octadecylalkyl groups. They recognized an improved enzyme activity due to an increase of hydrophobicity. This observation can be attributed to the structure of the lipase. As mentioned in chapter 2.2.3. the active site of the enzyme is covered by a lid which opens in the presence of hydrophobic solutions or interfaces resulting into a more accessible active site.[52, 209]

Amination with 3-aminopropyltrimethoxysilane (APTMS) of pre-synthesized silica particles followed by activation with GA and immobilization of enzymes, is a popular route to end up with a covalent attachment between enzyme and silica support (figure 2-15, B). NAZARI ET AL. reported the immobilization of a subtilisin protease using this approach. The immobilized protease was relatively stable in a storage test over 40 days.[210] Likewise YANG ET AL. immobilized lipase on an amino-modified silica gel. Additionally, GA was used to form CLEAs with immobilized and physically adsorbed lipase (figure 2-15, C). Characterizing and testing those three strategies (covalent attachment, covalent attachment plus cross-linking and CLEAs), they concluded that forming enzyme aggregates without further immobilization results in highest stability and activity.[211] The formation of CLEAs is a recently developed strategy driven by ROGER SHELDON to fix enzymes into mesoporous materials. On one hand stabilization effects for multimeric enzymes can be observed, but on the other hand there is a risk of denaturation due to crosslinking.[207] KIM ET AL. crosslinked lipase and α-chymotrypsin in mesoporous silica using GA. As a result, leaching was prevented and the enzyme stabilities were increased compared to the physically adsorbed ones.[212]

Compared to the other strategies, one-pot synthesis of mesoporous NP containing enzymes (figure 2-15, D) is more challenging and less used. The direct encapsulation of lipase, laccase and HRP into mesoporous silica is reported by SANTALLA ET AL. They identified the hydrophobicity of the enzymes´ surface as a significant parameter influencing the *in-situ* synthesis.[208]

2.6.3. Mesoporous Silica Nanoparticles in Detergent Applications: Open Research Questions

Enzyme encapsulation and immobilization using mesoporous silica NP is as mentioned before an extensively studied and established technology to improve the stability of enzymes. Nevertheless, like in the case of enzyme-polymer conjugates (chapter 2.5.), the number of publications dealing with an application regarding detergents is very limited.

IBRAHIM ET AL. reported the immobilization of protease onto rattle-type magnetic core/mesoporous shell silica nanoparticles. Firstly, they compared physical adsorption and covalent attachment of non-functionalized and amino-functionalized NP. Protease was attached using the homobifunctional crosslinker GA to activate the amino-functionalized NP. Covalent attachment of the enzyme performed better in immobilization yield (physical adsorption: 30%, covalent attachment: 90%). Secondly, they tested protease stability against temperature, organic solvents, surfactants and detergents. For investigating stability of free and immobilized protease against a selection of nonionic, cationic and anionic surfactants, they stored the enzyme at 40 °C for 1 h. In addition, they stored protease in several commercial liquid laundry detergents with a final detergent concentration of 1% for 24 h at room

temperature. In all storage tests the immobilized enzyme showed a higher stability compared to the free enzyme.[213] It should be said that the detergent concentrations chosen of IBRAHIM ET AL. are nowhere near realistic. A concentration of 1% is very diluted. Additionally, enzyme stability is only studied over a very limited period – maximum of one day. Even in this time frame, the residual activity of immobilized protease drops down and is not preserved.

The immobilization of protease on silica NP for an application in powder detergents is reported by SOLEIMANI ET AL. Thereby, about 80% of the enzyme are physically adsorbed on not further described silica NP. Over a period up to twelve weeks, the cleaning efficiency towards protein soil removal on cotton fabrics is investigated. SOLEIMANI ET AL. observed an increased cleaning efficiency and stability of the immobilized enzyme.[214] In this publication long-term experiments are described. However, SOLEIMANI ET AL. performed those experiments in powder detergents where stabilization effects are easier to achieve than in liquid detergents.

Accordingly, the stability of immobilized enzymes using mesoporous silica NP in liquid detergents is not studied under realistic conditions for a long-term. This work investigates this topic. Regarding the open research question, it will be tested if mesoporous silica nanoparticles can meet the requirements of detergent application. Is it possible to synthesize a silica material that is stable in a laundry formulation and shows a stabilizing effect on the detergent enzymes? In this respect the question arises whether the post synthesis loading of the enzyme into the particle pores occurs successfully. And it has to be considered that encapsulation and stabilization of the enzyme is not sufficient, the enzyme has to be well released at the right time – in the beginning of the washing process. With regard to the stabilizing, two different techniques to keep the surfactants aloof from the encapsulated enzymes are tested and compared: electrostatic repulsion and hydrophobic gating. Both techniques are explained in the following section.

2.6.4. Mesoporous Silica Nanoparticles in Detergent Applications: Own Strategy

The general approach is displayed in figure 2-16. Firstly, silica NP batches with different pore sizes are prepared. The surface of the materials prepared bear hydroxy groups resulting in a pH responsive behavior.[215] In that way a negatively charged surface will be present under detergent conditions (pH > 8) which can result into a repulsion of likewise negatively charged surfactant molecules. Thus, surfactants should be excluded and kept from enzymes that are encapsulated in the mesoporous NP via physical adsorption (figure 2-16 A).

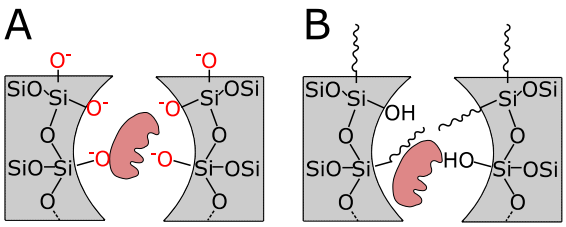


Figure 2-16: Approach for encapsulation of enzymes into mesoporous silica NP. Physical adsorption of the enzyme and exclusion of the surfactants via electrostatic repulsion (A) and hydrophobic gating (B).

Secondly, silica NP with varying pore sizes and wetting-properties will be prepared. For the adjustment of wetting-properties, the silica surface is functionalized with long-chained alkyl silanes. The introduction of alkyl silanes is expected to result in an increased hydrophobicity that can exclude water and relating thereto in a hydrophobic gating towards detergent ingredients like surfactants (figure 2-16 B). Again, the enzyme is intended to be encapsulated using physical adsorption. Provided that the enzyme can be encapsulated in the pores, this strategy has several benefits for a detergent application. Using adsorption, the enzyme is not chemically modified, and it can be assumed that the enzyme activity will remain unchanged. Additionally, a complete enzyme release during the washing process without disruptive silica fragments covalently attached to enzyme is possible. Despite the advantages, enzyme leaching can occur and lead to an enzyme activity loss. For a successful adsorption, it is estimated that the ideal pore diameter will be about 5 nm, which is slightly larger than lipase and small enough to avoid leaching. The location of the enzyme after immobilization, whether the enzymes is inside the pores or adsorbed externally, will be determined using different characterization methods. Measuring the protein content of the continuous phase using standard protein concentration assay is one method to calculate the enzyme encapsulation efficiency. To determine further the location of the enzymes indirect techniques like thermogravimetric analysis (TGA), Fourier transform infrared spectroscopy (FTIR) and nitrogen adsorption are used.[216-217] A direct visualization is possible using fluorescence microscopy with a fluorescent dye to label the enzymes.[218]

Expected Challenges

Working with the method of physical adsorption for enzyme encapsulation and stabilization is accompanied by the following expected challenges:

- Low encapsulation efficiencies in the pores of the silica nanoparticles

  Countermeasure: adjusting of pH value so that the hydroxy groups are deprotonated, and the enzymes positively charged (pH value > pI enzyme) → attractive interactions between enzyme and silica surface

- Enzyme leaching after a successful encapsulation

  Countermeasure: keeping the pore diameter only slightly larger than the enzyme
- Accessibility for large substrates to the encapsulated enzyme

  Countermeasure: enzyme release due to the large water quantity that influxes during the washing process (osmotic pressure release)

2.7. Metal-Organic Frameworks

The fourth strategy – metal-organic frameworks – described here belongs to the strategies "enzyme immobilization" and "enzyme encapsulation" (chapter 2.3., figure 2-6 E and F). Firstly, a general overview of metal-organic frameworks is given (chapter 2.7.1.). Secondly, the combination of metal-organic frameworks and enzymes is described. Here, recent developments in this area are mentioned (chapter 2.7.2.). Afterwards, the open research question is asked (chapter 2.7.3.) and finally, the strategy used is stated (chapter 2.7.4.).

2.7.1. Metal-Organic Frameworks: General Introduction

The term *metal-organic framework* (MOF) has been used for the first time from OMAR YAGHI in 1995 and designates hybrid materials consisting of inorganic units that form, together with an organic linker, a one-, two- or three-dimensional scaffold.[219] Interactions between organic and inorganic units results from coordinative bonds between a Lewis acid (metal cation) and a Lewis base (organic linker).[220] In general, MOFs are characterized by a highly ordered structure with pores and subsequently a large surface area.[221] Furthermore, MOFs show a high thermal (at least 300 °C) and chemical stability. Consequently, they are insensitive to extreme pH values and impervious to organic solvents.[222] OMAR YAGHI, as one of the pioneers in this area, demonstrated that it is possible to vary the pore size by the use of linkers with different length. His group synthesized a series of MOFs with disparate organic groups, consistent framework topology and varied the pores from 3.8 to 28.8 Å.[223] In addition, MOFs can be modified post-synthetically. INGLESON ET AL. functionalized an isoreticular metal–organic framework (IRMOF-3) with salicylaldehyde to end up with an immobilized ligand that can complex vanadium ions.[224]

It is possible to produce MOFs with almost all transition metals and many of the main group elements and thus a large variety of different MOFs exist. In most cases oxygen or nitrogen ligands like multifunctional carboxylates (O donor) or pyridine derivatives (N donor) are used as linkers. Sulfonates or phosphonates are more seldom.[225] The synthesis takes place in polar solvents like dimethylformamide (DMF) under solvothermal conditions at high temperatures and pressures. To remove solvents out of the pores, synthesized MOFs are treated in vacuum at elevated temperatures.[226] With regard to the described properties, MOFs are predestined for applications in gas storage,[227-228] separation processes[229] and catalysis.[230-231] The immobilization or rather encapsulation of enzymes in metal-organic frameworks is of particular interest for applications regarding biocatalysis.[232-233] Within a heterogeneous catalysis of a chemical reaction by an enzyme the product separation without enzyme contamination is facilitated. Of interest is the retained enzyme activity and stereoselectivity as well as the possible reuse of enzymes combined with MOFs. In

consequence of the immobilization in MOFs, many enzymes show an enhanced stability against temperature and organic solvents.[233] Besides the large field of biocatalysis, enzyme in MOFs are used as biosensors.[234-235] Strategies to immobilize or encapsulate enzymes into metal-organic frameworks are shown in the following section (chapter 2.7.2.).

2.7.2. Metal-Organic Frameworks and Enzymes

A broad range of solid supports – like hydrogels, sol gels, porous or non-porous inorganic supports – have been investigated for enzymes. These supports are reported to have various disadvantages like very low protein loading in non-porous systems or enzyme denaturation in sol gels. MOFs have properties that are ideal for enzyme immobilization or encapsulation. The frameworks possess a large surface area that can be modified and show a high chemical and mechanical stability.[236] In comparison with conventional enzyme hosts like mesoporous silica, MOFs stand out with an extremely high porosity and internal surface area that is adjustable as well as a tunable pore size, an excellent dispersity and modifiable organic linkers.[232, 237-239]

Up to now four different strategies are known to combine an enzyme with a metal-organic framework.[239] In figure 2-17 those strategies are displayed and a further distinction between a presynthesized MOF and co-precipitation is pointed out.

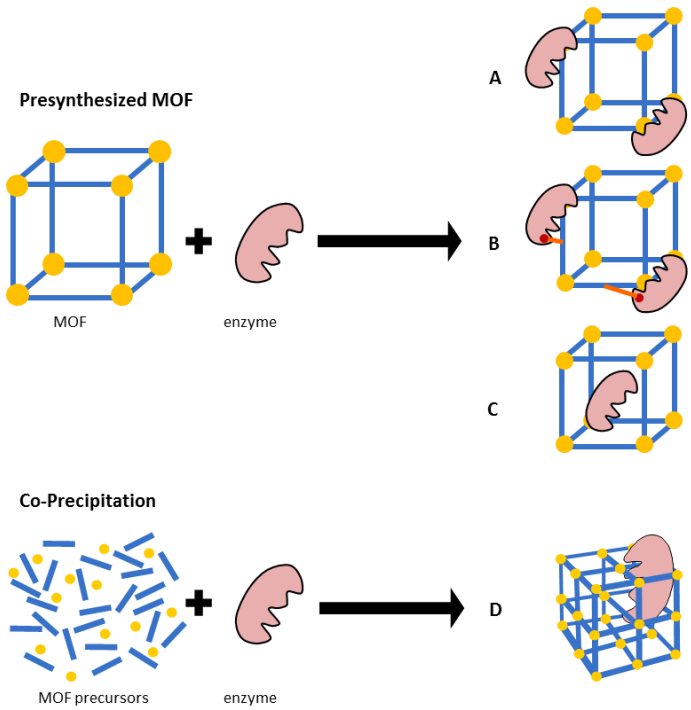


Figure 2-17: Illustration of the four different strategies to combine metal-organic frameworks and enzymes. Presynthesized MOF: adsorptive onto the MOF surface (A), covalent onto the MOF surface (B), encapsulation into the MOF pores (C). Co-precipitation: assembly of the enzyme into the MOF (D).

If the metal-organic framework is synthesized prior enzyme contact, it can be chosen between three different possibilities to immobilize the enzyme. The enzyme can remain outside the pores and attach adsorptively (figure 2-17 A) or covalently (B) to the surface of the presynthesized MOF. If the pores of the MOF are large enough, the enzyme can diffuse into the pores and is in this way encapsulated (C). Most of the existing MOFs have micropores (<2 nm) and are too small for an encapsulation of enzymes, which have an average hydrodynamical diameter of 3 to 5 nm.[240] MOFs that possess pores of sizes lager than 2 nm are called meso-MOFs. Alternatively, it is possible to co-precipitate enzymes and MOFs, which means that the framework is build up around the enzyme (figure 2-17 D). Co-precipitation is based on the interaction between amino acids and cations like Zn^{2+} or Ca^{2+}, which can result in biomineralization. Non-covalent interactions between the enzyme and the organic linker play a major role in the co-precipitation method.[241] Using this strategy, it is necessary to operate with aqueous systems for the synthesis. On the contrary, the other three strategies offer the possibility to synthesize the MOF under enzyme denaturing conditions like organic solvents or high temperatures.[239] Using the combination of enzymes and metal-organic frameworks is referred in most cases to the presynthesized MOF (figure 2-17 A-C). Co-precipitation of enzyme and MOF precursors is less utilized (figure 2-17 D). CHEN ET AL., for example, encapsulated horseradish peroxidase into a presynthesized porous coordination network (PCN; PCN-33(Fe)) (figure 2-17 C). The MOF used belongs to the meso-MOFs with a pore size of about 6 nm. Through this system, a biosensor with improved acidic pH and thermal stability was obtained.[242]

The adsorptive immobilization of fluorescein isothiocyanate (FITC)-labelled trypsin on various nanoporous MOFs for application in biocatalysis is studied by LIU ET AL. (figure 2-17 A). The MOFs tested are not chemically modified on their surfaces and the adsorbed enzyme shows a catalytic activity similar to free trypsin.[222] Utilizing the same enzyme, SHIH ET AL. linked trypsin covalently to the surface of two different chromium-based *Matériaux de l'Institut Lavoisier* (MIL) MOFs (MIL-101, MIL-88B and MIL-88B-NH_2) (figure 2-17 B). Free carboxylate groups of the integrated terephthalic acid were activated with dicyclohexylcarbodiimide (DCC) to enable a nucleophilic attack of the amino groups of trypsin. As a result, the proteolysis performance of trypsin is enhanced for an application as biocatalyst in proteomics analysis. A diffusion of trypsin into the MOF pores is in both examples prevented by the size of trypsin that is more than three times larger than the pore size of the MOFs tested.[243]

For synthesis under aqueous conditions using co-precipitation zeolitic imidazolate framework (ZIF) and especially ZIF-8 are most frequently used (figure 2-17 D). ZHANG ET AL. embedded Glucose oxidase into ZIF-8 for an application in electrochemical biosensing. Via the *in-situ* entrapment they encapsulated 89% of the enzyme and proved an enhanced enzyme stability towards high temperature (90 °C), organic solvents (acetone) and storage in buffer.[244]

Most recent publications trend towards the combination of MOF and silica encapsulation. For instance, CUI ET AL. co-precipitated in a first step catalase, 2-methylimidazole (HmIm) and zinc nitrate to form ZIF-8. In a second step the group encapsulated the catalase/ZIF-8 nanocrystals into a large mesoporous silica layer. As a result, about 80% enzyme activity were retained, and the enzyme showed an increased stability against proteolysis and extreme pH values. The encapsulated and immobilized catalase shows after one-hour incubation with trypsin an activity of 60%, whereas the free enzyme possesses a residual activity of 15%. The envisaged application for this MOF type is in the biocatalysis.[245]

Presynthesized MOFs and Co-Precipitation

In consequence of enzyme encapsulation or immobilization in MOFs, the residual enzyme activity can be reduced. This can be traced back to diffusion limitations due to a small channel or a blocked active site.[246-248] Enzymes immobilized or encapsulated in metal-organic frameworks which are described in literature implement in most cases small molecule substrates. Catalase or cytochrome C, for instance, are widely spread model enzymes that have hydrogen peroxide as substrate.[245, 249] Nevertheless, the difficulties of mass transfer between substrate and enzyme´s active site are a literature known phenomenon. It can be lessened if the enzyme is immobilized on the surface of a MOF and not encapsulated in a pore (figure 2.17 A+B). However, adsorption is difficult in solutions with high ion strength like detergents and covalent attachment can result in a decreased enzyme activity. Therefore, a more appropriate method for a detergent application might be co-precipitation with a high amount of enzyme that is embedded on the MOF surface. Thereby a high enzyme to MOF ratio should have a positive impact on enzyme recovery on the surface.

In addition, if the MOF is co-precipitated around the enzyme in an aqueous medium, problems regarding stability in water do not occur. Working with a presynthesized MOF, it is necessary to ensure that the MOFs are stable in water. Due to the non-covalent interactions between metal cation and organic linker, MOFs can be deconstructed in water.[243, 250] Water stability can be enhanced by the use of trivalent metals.[239] FENG ET AL., for instance, used PCN-333 with trivalent iron and aluminum and observed a stability in water in a broad pH range.[251] Another possibility to enhance the stability is the use of covalent-organic frameworks (COFs) with stable covalent bonds, as their name suggests.[252-254] However, the increased stability can become a problem when the enzyme release is prevented.

Further advantages of co-precipitation are higher enzyme loadings and a reduced enzyme leakage. GASCÓN ET AL. compared the post-synthesis and the *in-situ* enzyme loading in MOFs and find out that using co-precipitation, the enzyme loading is 50% higher and the enzyme leaching significantly reduced.[255] It can also be expected that enzymes within a co-precipitated MOF are more protected from denaturing conditions due to a framework that is built to match for the enzyme. The pores of

presynthesized MOFs are large, the enzyme has freedom of movement and denaturing agents can enter the pore as well.

Expected advantages (X) and disadvantages (X) of using a presynthesized MOF and co-precipitation to combine MOFs and enzymes are summed up in table 2-3.

Table 2-3: Summary of expected advantages and disadvantages of using presynthesized MOFs and co-precipitation for enzyme immobilization and encapsulation. Uncritical issues are marked with a green cross (X) and critical with a red cross (X).

Issue	Presynthesized MOF	Co-precipitation
Water stability	X	X
Activity of enzyme (accessibility of substrate)	(X)	X
Enzyme encapsulation	(X)	X
Enzyme leakage	X	X
Enzyme protection	X	X

2.7.3. Metal-Organic Frameworks in Detergent Applications: Open Research Questions

In general, the use of MOFs in enzyme-related applications is not yet well established and the topics interest has arisen in recent years. This is clearly illustrated by a view on the number of publications containing the keywords “metal organic frameworks enzyme” per year (web of science). The histogram shown in figure 2-18 has an exponential course with twelve publications in 2012 and at least 150 publications in 2018 (state October 8th).

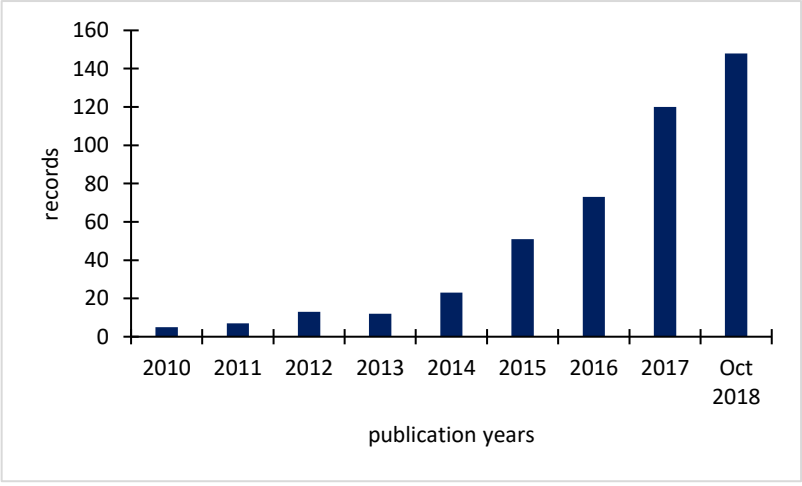


Figure 2-18: Histogram showing the number of publications per year regarding “metal organic frameworks enzyme” (Web of Science).

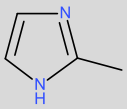
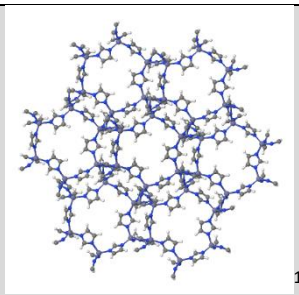
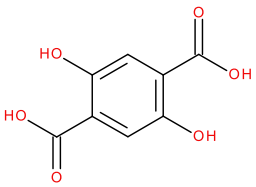
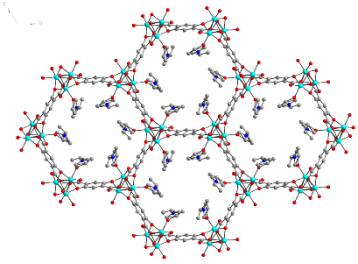
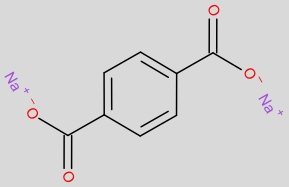
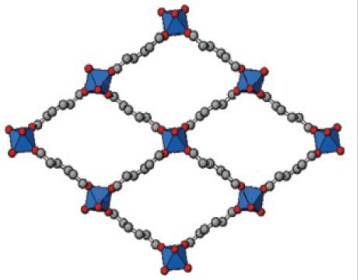
It is the ambition to immobilize or encapsulate enzymes into MOFs with a view to protect the biomolecule from a denaturing external environment like elevated temperature, surfactants or protease while maintaining the accessibility of the active site for the substrate. Observed enhanced enzyme stabilities in combination with MOFs in literature are restricted to organic solvents,[244] urea,[256] temperature[257] and proteolysis.[245] To the best of my knowledge enzyme stability in a detergent formulation or a single surfactant has not been described so far. A positive impact on enzyme stability in detergents should have the increased rigidity of the enzyme due to the immobilization. The metal-organic framework confines the changes of the enzyme structural conformation and the ability of unfolding, combined with a loss of function, should be reduced. LIAO ET AL. embedded catalase into ZIF-8 and ZIF-90 and observed a greater stability against urea and elevated temperature.[256] Further advantages of MOFs for enzyme stabilization in detergents are the previous described reduction of enzyme aggregation and dissociation, as well as accessibility to proteolysis (chapter 2.6.2.).

Publications dealing with co-precipitation of enzymes and MOF precursors are primarily limited to the formation of ZIF-8 as MOF. In the present thesis, it is studied if detergent enzymes can be embedded into MOFs that are not investigated for an enzyme application so far. The question whether the enzymes survive the encapsulation process is pursued as well as the amount of residual activity. Further the stability of MOFs in liquid detergent formulations is unknown and therefore studied. Finally, it is checked which of the MOFs tested shows the best enzyme stabilization properties against a liquid detergent and proteolysis.

2.7.4. Metal-Organic Frameworks in Detergent Applications: Own Strategy

On the basis of the expected advantages and disadvantages as well as of preliminary studies (data not shown) it is decided to use the co-precipitation strategy to combine MOFs and enzymes. Using co-precipitation three different metal-organic frameworks have been tested to immobilize and encapsulate protease, α-amylase and lipase. These MOFs are ZIF-8, MOF-74 and MIL-53. Table 2.4 shows an overview of the MOFs synthesized and tested.

Table 2-4: Overview of the different MOFs used for co-precipitation with lipase.

Name	Organic Linker	Metal Ion	Crystal Structure
ZIF-8	2-methylimidazole 	Zn^{2+}	 [1]
MOF-74	2,5-dihydroxy-terephthalic acid 	Zn^{2+}	
MIL-53	Terephthalic acid 	Al^{3+}	

As mentioned before, the usage of ZIF-8 is very common due to its facile and rapid synthesis in water.[258-259] Zn^{2+} is coordinated by 2-methylimidazole (HmIm) to form a rhombic dodecahedral structure.[102] By varying the molar ration between metal ion and linker and the water content, crystallization,[260] porosity[259] and surface area[261] can be influenced. In total, ZIF-8 is very suitable for enzyme encapsulation, as shown by the numerous examples in literature.[262-264]

MOF-74 can be built up with a variety of metals – Mn, Co, Ni, Mg, Zn – using the same organic linker: 2,5-dihydroxyterephthalic acid (DHTP).[265-269] The metals are coordinated to five oxygens from DHTP and one solvent molecule and the MOF exhibits a honeycomb motif.[270] Worth mentioning is the high density of metal sides and the high stability of MOF-74. Not all members of the MOF-74 family are stable in water. The crystal structure of Ni-MOF-74, for instance, is destroyed by water while with magnesium the structure remains.[271] Therefore, MOF-74 is usually synthesized in organic solvents without or with a small presence of water.[266, 272] SÁNCHEZ-SÁNCHEZ ET AL. described the synthesis of

[1] http://www.chemtube3d.com/solidstate/MOF-ZIF8.htm

MOF-74 at room temperature using the disodium salt of DHTP and zinc acetate.[273] Though, the combination of MOF-74 with enzymes is so far described in one publication using DMF as solvent for the encapsulation of β-glucosidase into Mg-MOF-74.[255] Here, MOF-74 will be synthesized using co-precipitation in the presence of enzymes and with water as solvent.

Not for enzyme applications but in general, MIL-53(Al) is a very well-studied MOF that is commercially available under the name Basolite® A100. [AlO4(OH)2] octahedrons are coordinated by the carboxy groups of terephthalic acid to a 3D structure. Additionally, the hydroxy groups are corner connected. MIL-53(Al) is characterized by one of the highest thermal stabilities in the presence of oxygen (> 500 °C). Normally, MIL-53 is synthesized hydrothermally at 220 °C in water for three days.[274] However, SÁNCHEZ-SÁNCHEZ ET AL. synthesized Al-MIL-53 in water and at room temperature using the disodium salt of terephthalic acid and aluminum nitrate in a few hours. The crystals obtained were extraordinary small with a high external surface area.[273] The same group investigated the co-precipitation of β-glucosidase and MIL-53, this is the only publication regarding Al-MIL-53 and enzymes.[255]

Expected Challenges

Alongside the benefits brought about by enzyme immobilization or encapsulation in MOFs, drawbacks can also occur. Expected challenges are listed below:

- Enzyme do not survive unscathed the encapsulation process
  Countermeasure: physiological conditions (aqueous, widely neutral pH) during synthesis
- Enzyme leaching after successful embedding
- Accessibility for large substrates to the embedded enzyme
  Countermeasure:
    high enzyme to MOF ratio for enzyme recovery on the MOF surface;
    enzyme release due to the large water quantity that influxes during the washing process (osmotic pressure release)

3. Aims and Objectives

The aim of this work is the development and comparison of four different enzyme stabilizing systems (figure 2-8) that enable at best a long-term stability of enzymes in a liquid detergent formulation. More concretely, the stabilizing systems shall contribute to no or only hardly reduced residual enzyme activity after storage in a standard liquid detergent formulation over a period of four weeks at a temperature of 37 °C. In this way, three different, laundry application relevant enzyme types – a protease, α-amylase and lipase – are tested. Additionally, the stabilizing systems shall increase the stability of lipase in presence of protease. Firstly, the stabilizing systems – small molecules, enzyme-polymer conjugates, mesoporous silica nanoparticles and metal-organic frameworks – are tested regarding their suitability for a detergent application. Scientific issues relating to this topic will be clarified. Finally, all strategies are compared among each other.

It is known that **small molecules** can have a strong influence on the activity and stability of enzymes (see under chapter 2.4.). The four different small molecules of choice (acetylcholine, citric acid choline ester, triethyl citrate, acetyl triethyl citrate) contain at least one ester bond and should therefore interact with the lipase active site. A temporary block of the active site increases the rigidity of one of the most important enzyme parts. As a result, it is likely that lipase stability can be increased by the presence of substrate related small molecules. In contrast, an enhanced lipase stability against proteolysis is not expected. A small molecule occupies only a small area of the enzyme, while the main enzyme part is unaffected and easily accessible to proteases. In this case the enzyme's stability could be increase without a chemical modification of the target enzyme. This is a major advantage. Here, the small molecules without an irreversible inhibition represent the only strategy where no reduction of enzyme activity against stained clothes is expected. The high dilution during the washing process should release the small molecule and clear the lipase active site. For the other systems it is uncertain how accessible the active site is after addition and synthesis of the stabilizing formulation.

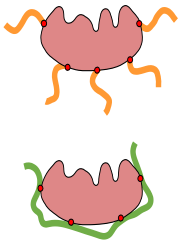
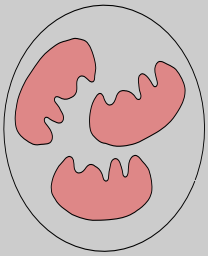
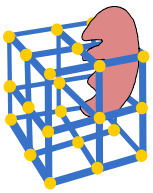
Enzyme-polymer conjugates are promising as a strategy to increase the stability of enzymes in liquid detergent formulations. With the conjugation via the amino groups the number of positive charges on the enzyme surface is decreased and coupled with the advantageous effect that electrostatic interactions between enzyme and the negatively charged anionic surfactants should be reduced. In addition, the conjugation with a polymer is reported to have in most cases no effect on the enzyme conformation[275] and even in contrary the rigidity of an enzyme is increased, which should retain the enzyme conformation despite presence of denaturing surfactants. The active site of an enzyme is shielded by the polymers and enzyme activity should be preserved.[276] Those possible effects should be enforced by the polysaccharides (maltodextrin and CMC) and especially by CMC, due to the negative charges in the side chain. They can form stabilizing salt bridges and lead to electrostatic repulsion with

other enzymes as well as anionic surfactants.[277] Furthermore conjugated enzymes should be protected against proteolytic degradation by proteases.[276] However, the performance of an enzyme can be reduced after conjugation due to a steric hindrance of the polymer chains.[278] It is quite possible that this could be the major problem for the application in a detergent, because of the poorly accessible large substrates immobilized on textile fibers.

Mesoporous silica nanoparticles and **metal-organic framework** are in direct competition, due to the method of enzyme encapsulation used in both strategies. Provided that the enzyme is encapsulated successfully in the silica NP as well as in the MOFs, both strategies give rise to the same benefits received by enzyme immobilization. Immobilized enzymes are less susceptible to aggregation, proteolysis,[279-280] or to a dissociation of the subunits in case the enzyme is a multimer.[281] Additionally, the enzyme structure is more rigid which enhances the stability as mentioned for the enzyme-polymer conjugates.[282-283] For mesoporous silica NP, two different techniques to keep the surfactants away from the encapsulated enzymes will be tested: Electrostatic repulsion and hydrophobic gating. Due to the enzyme encapsulation in mesoporous silica NP via physical adsorption, it is to be expected that a leaching problem can arise. The 1D channel structure can interact with enzymes without further modification only insufficiently.[284-285] The risk for leaching is diminished by the co-precipitation of the MOF precursors around the enzyme. Here, the pores should not be larger than the enzyme itself. In total, three different MOFs – ZIF-8, MOF-74 and MIL-53 will be synthesized by co-synthesis and investigated.

In table 3-1 expected strengths and weaknesses of each strategy as well as open questions are summarized.

Table 3-1: Expected strengths, weaknesses and open questions of each strategy tested for enzyme stabilization in a liquid detergent formulation.

Strategy	Expected strengths	Expected weaknesses	Open questions
Small molecules 	▪ No reduction of the residual enzyme activity ▪ Enhanced enzyme stability in a liquid detergent	▪ No lipase stabilization against protease	▪ Targeting of lipase possible? ▪ Influence on other enzymes (protease and α-amylase)?
Enzyme-polymer conjugates 	▪ Enhanced enzyme stability in a liquid detergent ▪ Enhanced stability of lipase against proteolysis	▪ Reduced residual enzyme activity due to conjugation	▪ Glycosylation or PEGylation? ▪ Accessibility of large substrates (stain) given?
Mesoporous silica NP 	▪ Enhanced enzyme stability in a liquid detergent ▪ Enhanced stability of lipase against proteolysis	▪ Reduced residual enzyme activity due to encapsulation ▪ Enzyme leaching ▪ Can be high encapsulation yields achieved?	▪ Electrostatic repulsion or hydrophobic gating? ▪ Accessibility of large substrates (stain) given?
Metal-organic frameworks 	▪ Enhanced enzyme stability in a liquid detergent ▪ Enhanced stability of lipase against proteolysis	▪ Reduced residual enzyme activity due to encapsulation ▪ Enzyme leaching	▪ MOF stability in a liquid detergent? ▪ ZIF-8, MOF-74 or MIL-53? ▪ Accessibility of large substrates (stain) given?

4. Materials and General Methods

In this chapter materials used (chemicals and instruments) are listed and general methods operated with are shown. Additionally, the theory behind those methods is explained briefly.

4.1. Materials

All chemicals and materials are used without further purification and demineralized water was used for all experiments. Purchased chemicals are listed in table 4-1.

Table 4-1: Tabular list of the chemicals used.

Chemical	Supplier	Order number	Other
Acetic acid	Bernd Kraft	05121	100%
Acetone	BASF SE		
Acetonitrile	BASF SE		
Acetylcholine chloride	Sigma-Aldrich	A6625	≥ 99%
L-alanine	Sigma-Aldrich	05129	≥ 99.5%
Aluminium nitrate nonahydrate	Sigma-Aldrich	237973	
α-amylase (lyophilized)	Sigma-Aldrich	A4551	*Bacillus licheniformis*
α-amylase (Termamyl 300L)	Sigma-Aldrich	A4862	*Bacillus licheniformis*
α-Glucosidase	Roche Applied Science	11626329103	Multifunctional
Bicinchoninic acid (BCA) protein assay kit	ThermoScientific	10741395	
Bicine	Sigma-Aldrich	B3876	≥ 99%
Boric acid	Sigma-Aldrich	B6768	
Calcium chloride dihydrate	Sigma-Aldrich	31307	
Choline chloride	Sigma-Aldrich	C7017	≥ 99%
Coomassie Brilliant Blue R250	Sigma-Aldrich	B7920	
2,5-Dihydroxyterephthalic acid (DHTP)	TCI	D3899	≥ 98%
Dimethylformamide (DMF)	BASF SE		
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	276855	anhydrous, ≥ 99.9%

Disodium ethylenediaminetetraacetate dihydrate (EDTA)	Bernd Kraft	06200	
ES1-M	BASF SE		Standard detergent formulation
Ethanol	BASF SE		
Ethylidene-blocked-4-nitrophenylmaltoheptaoside (EPS)	Roche Applied Science	10880078	
4 Formylphenylboronic acid (4-FPBA)	Sigma-Aldrisch	431966	≥ 95.0%
Gum Arabica	Sigma-Aldrich	51198	
Lipase (Lipolase 100L)	Sigma-Aldrich	L0777	*Thermomyces lanuginosus*
2-(*N*-morpholino)ethane sulfonic acid (MES)	Sigma-Aldrich	M3671	
3-(*N*-morpholino)propane sulfonic acid (MOPS)	Sigma-Aldrich	M3183	≥ 99.5%
mPEG-aldehyde	Sigma-Aldrich	JKA3039	5 kDa
mPEG-NHS	Biochempeg	MF001029-5K	5 kDa
Maltodextrin	Sigma-Aldrich	419699	
Methanol	BASF SE		
2-Methylimidazole (HmIm)	Sigma-Aldrich	M50850	
4-Nitrophenyl valerate	Sigma-Aldrich	N4377	
Peptide aldehyde	BASF SE		
Potassium hydroxide	Bernd Kraft	06215.2600	For analysis
Protease (PA1, lyophilized)	BASF SE		
Protease (Savinase 16.0L)	Sigma-Aldrich	P3111	*Bacillus sp.*
Polyethylenglycol	Merck Millipore	817007	6 kDa
Polyethylenglycol	Merck Millipore	821881	10 kDa
Polyoxyethylene(23)lauryl ether (Brij® L23)	Sigma-Aldrich	16005	

Polysorbate 20 (Tween®20)	Sigma-Aldrich	P2287	
SeeBlue® Plus 2 Pre-stained Protein Standard	ThermoFisher Scientific	LC5925	
Sodium acetate	Sigma-Aldrich	23218	
Sodium borate decahydrate	Sigma-Aldrich	B9876	≥ 99.5%
Sodium bromide	Sigma-Aldrich	229881	≥ 99.99%
Sodium citrate dihydrate	Sigma-Aldrich	71406	
Sodium chloride	Bernd Kraft	04160.3600	For analysis
Sodium cyanoborohydride	Sigma-Aldrich	42077	
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	71729	
Sodium format	Sigma-Aldrich	107603	97%
Sodium hydroxide	Sigma-Aldrich	306576	
Sodium hypochlorite	Bernd Kraft	07064.3010	13%
Sodium phosphate dibasic	Sigma-Aldrich	71640	anhydrous
Suc-Ala-Ala-Pro-Phe-*para*-nitroaniline (Suc-AAPF-pNA)	Bachem	4002299	
SYPRO Orange	Sigma-Aldrich	S5692	
Terephthalic acid	Sigma-Aldrich	185361	
Texapon N70	BASF SE		
Trichloroacetic acid (TCA)	Sigma-Aldrich	T6399	
Triethyl citrate (TEC)	Sigma-Aldrich	W308307	≥ 99%
Trifluoroacetic acid (TFA)	Sigma-Aldrich	302031	≥ 99%
Trinitrobenzenesulfonic acid (TNBS)	Sigma-Aldrich	P2297	5% (w/v) in water
Tris(hydroxymethyl)aminomethane (TRIS)	Sigma-Aldrich	33742	≥ 99.5%
Zinc acetate dihydrate	Bernd Kraft	12624	For analysis

Table 4-2 contains a compilation of the instruments used in the present thesis.

Table 4-2: Tabular list of the instruments used.

Instrument	Producer
5702 R	eppendorf
Minispin centrifuge	eppendorf
SPECTROstar®	BMG Labtech GmbH
Infinite M200Pro	Tecan Group AG
Excellence Plus Waagen	Mettler Teledo AG
Portamess 911	Knick elektronische Messgeräte
X-Cell ShureLock	Invitrogen
nanoDSC	TA instruments
Launder-o-meter	SDL Atlas
480 Lightcycler®	Roche
Prometheus NT.48	Nanotemper
Alpha 2-4 LSCplus	Martin Christ
HPLC	Agilent Technologies
Eclipse AF4 separation system	Wyatt Technology Corporation
Tecnai 12	Philips
Bruce	Memert
PXRD	Rigaku Miniflex 600

4.2. General Methods

General methods starting with the enzyme activity assays are described in this chapter. Additionally, conditions for enzyme performance tests and standardized measurements are specified. Further procedures used for enzyme stabilization (small molecules, enzyme-polymer conjugates, mesoporous silica nanoparticles and metal-organic frameworks) are shown, too.

4.2.1. Enzyme Activity Assays

This subsection deals with three enzyme activity assays – protease, α-amylase and lipase – performed in the present thesis. All three assays are absorption based.

Protease (according to STONER ET AL.[55])

Basis of the determination of protease activity is the cleavage of an artificial peptide substrate, Suc-Ala-Ala-Pro-Phe-*para*-nitroaniline (Suc-AAPF-pNA) (figure 4-1), resulting in the release of *para*-nitroaniline. The release of the dye is followed by measuring the absorption at 405 nm.

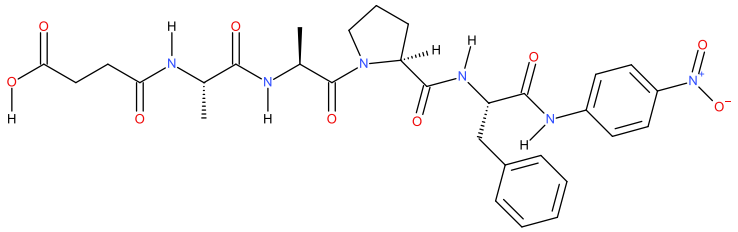


Figure 4-1: Structure of Suc-AAPF-pNA, the substrate of the protease assay.

For this assay a tenfold concentrated buffer with the following ingredients is prepared:

1 M TRIS	1% Brij® L23	pH=8.6	water

The compounds are dissolved in water, before the pH value at 30 °C is adjusted to 8.6 using a concentrated sodium hydroxide solution. To obtain the actual reaction buffer, the tenfold buffer is diluted to an onefold buffer using water. Using the onefold reaction buffer, standards for calibration and samples are diluted.

Suc-AAPF-pNA is dissolved in anhydrous DMSO with a concentration of 60 mg/mL and stored at -20 °C prior to usage. The starter solution consists of 88 Vol.-% water, 10 Vol.-% tenfold buffer and 2 Vol.-% of the 60 mg/mL substrate solution.

For running an assay, 50 µL enzyme solution (calibration and samples) are pipetted into a microtiter plate for a three-fold determination. Used microtiter plates are made of PS and have a flat bottom (Greiner, REF 655101). 100 µL starter solution are added to the 50 µL enzyme solution respectively.

Filled microtiter plates are placed immediately in the plate reader which is tempered to 30 °C and the measuring program is started. After an incubation of 180 seconds the plate is shaken with a frequency of 500 rpm for five seconds. Subsequently, the absorption measurement at 405 nm starts. Every minute over elf cycle in total the absorption is measured.

For analysis, measured values are corrected with the value of the blank sample (buffer, no enzyme). The slope of the samples is calculated as absorption per minute. Absorptions higher than 2.0 are outside the linear range and therefore neglected. Slopes are plotted against concentrations to obtain the calibrations curve. Using the linear equation, concentrations of the samples are determined.

In table 4-3 the protease activity assay is summarized.

Table 4-3: Key parameters of the protease activity assay.

Protease Activity Assay	
Buffer	Tenfold buffer: 1 M TRIS, 1% Brij® L23
pH value	8.6
Substrate solution	60 mg/mL Suc-AAPF-pNA in DMSO
Starter solution	88 Vol.-% water 10 Vol.-% tenfold buffer 2 Vol.-% substrate solution
Microtiter plate	50 µL enzyme 100 µL starter solution
Measuring program	Temperature: 30 °C Incubation: 180 s Shaking: 500 rpm for 5 s Absorption: 405 nm Cycle number / time: 11 / 1 min

α-Amylase (according to LORENTZ ET AL.[286])

Detection of α-amylase activity takes place via the measurement of the cleavage of the substrate Ethylidene-blocked-4-nitrophenylmaltoheptaoside (EPS; figure 4-2).

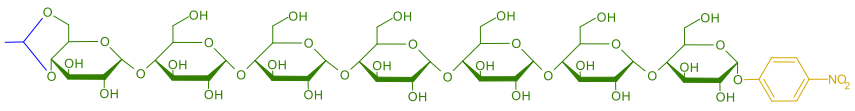


Figure 4-2: Structure of Ethylidene-blocked-4-nitrophenylmaltoheptaoside, the substrate of α-amylase assay.

In this assay, a second excess enzyme – α-glucosidase – is present. Hydrolysis of EPS by α-glucosidase is prevented by the ethylidene group until an internal substrate bond is cleaved by α-amylase. After this trigger the substrate is degraded by α-glucosidase to glucose and *para*-nitrophenol. The release of the dye is determined by measuring absorption at 405 nm and is directly proportional to the present α-amylase activity.

Operations of the enzyme activity assays are quite similar and not described further for α-amylase. In table 4-4 key parameters of the α-amylase assay are summarized.

Table 4-4: Key parameters of α-amylase activity assay.

α-Amylase Activity Assay	
Buffer	50 mM MOPS 50 mM sodium chloride 0.2% (v/v) Tween®20
pH value	8.5
Substrate solution	0.71 mg/mL α-glucosidase in buffer 18.2 mg/mL EPS in buffer
Starter solution	1:1-mixture of both substrate solutions
Microtiter plate	50 µL enzyme 50 µL buffer 50 µL starter solution
Measuring program	Temperature: 30 °C Incubation: 180 s Shaking: 500 rpm for 5 s Absorption: 405 nm Cycle number / time: 11 / 1 min

Lipase (according to PALACIOS ET AL.[287])

Lipase activity is determined using 4-nitrophenyl valerate as substrate (figure 4-3). Due to the cleavage by lipase *para*-nitrophenol is released and the absorption at 405 nm can be measured.

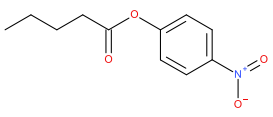


Figure 4-3: Structure of 4-nitrophenyl valerate, the substrate of lipase assay.

Operations of the enzyme activity assays are quite similar and not described further for lipase. In table 4-5 key parameters of the lipase assay are listed.

Table 4-5: Key parameters of lipase activity assay.

Lipase Activity Assay	
Buffer	100 mL TRIS (1 M, pH=8.0) 2 wt.-% gum arabica Up to 1000 mL
pH value	8.0
Substrate solution	40 mM 4-nitrophenyl valerate in DMSO
Starter solution	300 µL substrate solution 4700 µL substrate buffer (10 mM sodium acetate trihydrate, 0.086 wt-% Texapon®N70, pH=4.5)
Microtiter plate	50 µL enzyme 100 µL buffer 50 µL starter solution
Measuring program	Temperature: 30 °C Incubation: 120 s Shaking: 500 rpm for 5 s Absorption: 405 nm Cycle number / time: 11 / 1 min

4.2.2. Standardized Measurements

Standardized measurements that are used for all stabilization strategies in the present thesis are described in this subsection. This also includes further enzyme characterization methods like assays for protein concentration or differential scanning calorimetry and general analytic tools such as powder X-ray diffraction or transmission electron microscopy.

UV Assay

The simplest way to determine the protein concentration is measuring the absorption at 280 nm. At this, the π-π* absorption of the aromatic amino acids – phenylalanine, tyrosine and tryptophan – is

used. An absorption of 1 at 280 nm is equated with a protein concentration of 1 mg/mL. However, this method can be easily influenced by disruptive factors and the amount of aromatic amino acids varies widely from protein to protein.[288] In the present thesis a calibration curve of the enzyme measured is used for the determination of protein concentration. 150 µL of enzyme solution (calibration and sample) are filled into a UV light-transparent microtiter plate (Corning, 3635) and the absorption is measured at 280 nm.

Bicinchoninic Acid Assay (BCA Assay)

The bicinchoninic acid assay (BCA assay) is a method to determine the concentration of a protein in solution. Basis of the BCA assay is the reducing effect of the amino acid residues of cysteine, tyrosine and tryptophan in an alkaline environment in presence of sodium tartrate. As result Cu^{2+} is reduced to Cu^{+} and one Cu^{+} forms together with two bicinchoninic acid molecules a violet colored complex. This complex is water-soluble and can be quantified by absorption measurement at 562 nm.[289] In figure 4-4 the underlying complex formation is displayed.

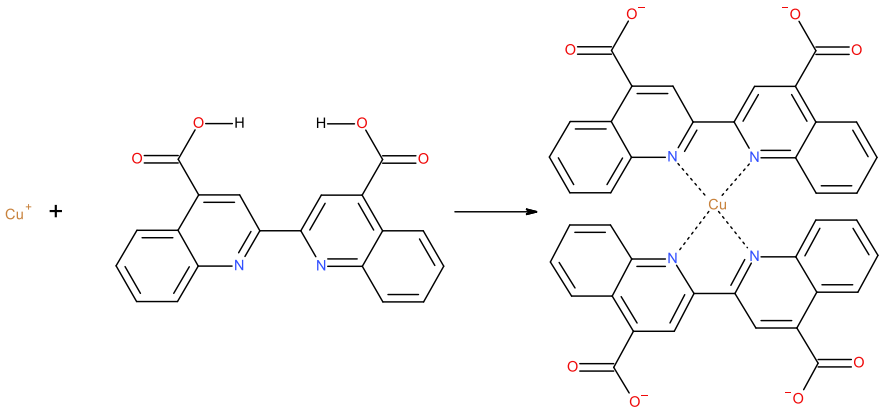


Figure 4-4: Formation of the chelate complex consisting of one Cu^{+} ion and two bicinchoninic acid molecules.

For the implementation an assay kit from ThermoScientific (23225) has been used. This kit contains BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1 M sodium hydroxide) and BCA reagent B (4% copper sulfate). A 50 to 1 mixture of reagent A to B forms the starter solution. 25 µL enzyme solution (calibration and samples) and 200 µL starter solution are filled into a microtiter plate (PS, flat bottom, Greiner, REF 655101). The plate is covered and incubated for 30 minutes at 37 °C. After incubation and cooling down to room temperature, the absorption is measured at 562 nm.

Trinitrobenzenesulfonic Acid Assay (TNBS Assay; according to HABEEB ET AL.[290])

The trinitrobenzenesulfonic acid assay (TNBS assay) is used to determine the amount of free and accessible amino groups of an enzyme. Free amino groups form Trinitrophenyl derivatives with TNBS which can be detected by measurement of the absorption at 420 nm (figure 4-5).[290]

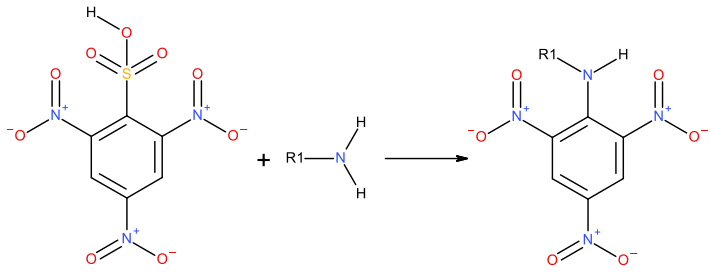


Figure 4-5: TNBS assay for calculation of free amino groups.

A bicine buffer (20 mM, pH=8.5) is used for the dilution of calibration samples and measured samples. For calibration, the amino acid L-alanine, which contains one free amino group, is diluted to several concentrations. For this assay, molar concentrations of calibration (L-alanine) and samples (enzyme) are compared. Starter solution is TNBS with a concentration of 0.1% (w/v). 75 µL calibration or sample solution and 37.5 µL starter solution are filled into a microtiter plate (PS, flat bottom, Greiner, REF 655101). The plate is incubated for one hour at 37 °C. After cooling down to room temperature, the absorption is measured at 420 nm. Using the calibration curve, the amino concentrations of the enzyme samples are calculated. The amount of accessible amino groups is obtained by division of the calculated amino concentration by the enzyme concentration.

Dialysis

Enzyme dialysis is performed for 24 hours at room temperature against water by using SnakeSkin Dialysis Tubing (ThermoFisher, 68100) with a 10 kDa cut-off.

Nano Differential Scanning Calorimetry (nanoDSC)

Changes of physical properties of a biopolymer sample along with temperature against time is determined by differential scanning calorimetry (DSC). Using a DSC instrument, the specific heat of a system is measured as function of temperature at a given scan rate relative to a reference solution. Due to material transitions, a heat quantity is radiated or absorbed by the sample during temperature changes. In general, two types of DSC instruments exist: heat-flux and power-compensated DSCs.

Within a heat-flux DSC, sample and reference are placed on a thermoelectric disk surrounded by a furnace which is heated. The heating rate is linear and due to the heat capacity of the sample, a temperature difference between sample and reference occurs and is measured. Two separated furnaces for sample and reference are used in a power-compensated DSC. Here, sample and reference are maintained at the same temperature. Thereby, the differential power required to maintain the temperature of a sample at the same value as the reference is quantified. Resulting in an output data that shows the power as a function of time. For more thermodynamic significance, the power is converted to the molar heat capacity and the time to the temperature.[291]

For biomolecules in aqueous solution an equilibrium between their native, folded conformation and denatured, unfolded state exists. The extend of Gibbs free energy (ΔG) is basis of the native conformation stability. Generally, the more negative the Gibbs free energy, the more favored the native conformation and the more stable the protein. In the unfolding process the stabilizing forces are outweighed by entropy at a certain temperature, resulting in a denatured protein.[292] Figure 4.6 shows a typical thermogram for a protein.

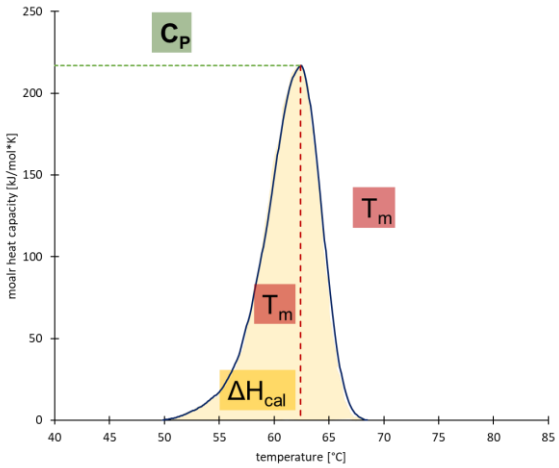


Figure 4-6: Typical thermogram of a nanoDSC measurement.

The transitions midpoint T_m corresponds to the temperature where half of the protein owns its native conformation, while the other half is already denatured. A higher T_m value is combined with a more stable protein under the tested conditions. The molar heat capacity (C_p) is obtained through the peak´s height. By integration the area under the thermogram peak, the calorimetric enthalpy (ΔH_{cal}) can be determined. The total heat absorbed by the sample due to transition is cumulated in this enthalpy. All thermodynamic parameters can be summarized to the Gibbs Helmholtz expression to calculate the Gibbs free energy (equation 1).[293]

$$\Delta G°(T) = \Delta H - T \cdot \Delta S$$

$$\Delta G°(T) = \Delta H\left(1 - \frac{T}{T_m}\right) + C_p[(T - T_m) - T \cdot \ln\left(\frac{T}{T_m}\right) \quad (1)$$

The used DSC from TA instruments (nanoDSC) is power-compensated and designed for liquid samples. For a measurement, sample (contains the protein of interest with a concentration of at least 1 mg/mL) and reference solution (contains only the solvent) are degassed for 20 minutes, before 1 mL is added respectively into a deep-well plate of the autosampler. The autosampler is tempered to 4 °C and fills the solutions into two measuring cells (platinum). Sample and reference cell go through the same temperature program up to 130 °C with a heating rate of 1.5 K/min. For analysis, the software package nanoAnalyze (Version 3.4.0, TA Instruments) is used. Measured thermograms are baseline corrected by blank measurements.

Differential Scanning Fluorimetry (DSF)

Differential scanning fluorimetry (DSF) or thermal shift assays is used to determine the change of thermal denaturing temperature of a protein. The underlying principle of DSF is shown in figure 4-7. In general, a hydrophobic dye like SYPRO Orange or 8-anilinonaphthalene-1-sulfonic acid (1,8-ANS) is added to the protein under varying conditions. In water at room temperature the protein is folded and hardly no interaction between dye and protein occurs. In addition, water quenches the fluorescence strongly. The samples are heated, the protein starts to unfold, and the hydrophobic parts of the protein that can interact with the dye are exposed. As result of binding on the hydrophobic surfaces, the fluorescence signal of the dye increases.

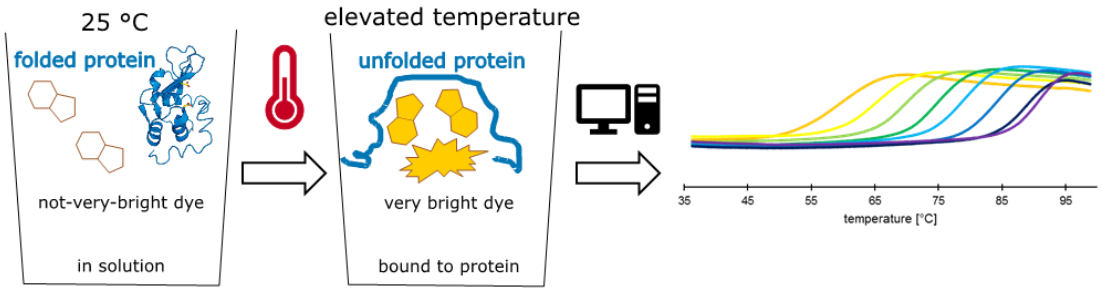


Figure 4-7: Principle of differential scanning fluorimetry. Due to an increase in temperature the protein starts to unfold, and a hydrophobic dye can bind to the hydrophobic protein parts. The resulting increasing fluorescence signal can be analyzed regarding the inflection point and the T_m value of the condition tested is determined.

Measuring the fluorescence signal sigmoidally shaped stability curves are obtained (figure 4-7). The value of the inflection / midpoint can be determined. Like nanoDSC measurements, at this temperature (T_m value) half of the protein own their native conformation, while the other half is already denatured. The higher the T_m value the more stable the protein under the tested conditions.

For the purposes of a DSF assay, a protein stock solution with a protein concentration of 1.5 mg/mL is prepared in water. 350 µL of this stock solution are added to 35 µL SYPRO Orange. Respectively 3 µL of the protein-dye mixture are placed in one well of 96-well polymerase chain reaction (PCR) plate (polypropylene, white, Corning Axygen® PCR-96-LC480-W). Subsequently, 97 µL of the solution tested are added and mixed with enzyme and dye. The PCR plate is covered and placed in a µPCR machine (Roche 480 Lightcycler®). Heating program with a heating rate of 1.5 K/min starts immediately up to a temperature of 95 °C. For analysis, the measured fluorescence signal as function of the temperature is plotted in excel. The first derivative of the plots can be calculated, and the inflection point becomes a peak maximum (T_m value) that can be easily read out.

Nano Differential Scanning Fluorimetry (nanoDSF)

In contrast to the DSF assay described in the previous section, nano differential scanning fluorimetry (nanoDSC) process without a dye. The nanoDSF method takes advantage of the intrinsic fluorescence of a protein. Tyrosine and especially tryptophan have fluorescent properties where the excitation and emission wavelength depend on the environment present. Therefore, a differentiation between a folded and an unfolded protein is possible. The ratio of fluorescence intensity at 350 nm (unfolded) and at 330 nm (folded) can be plotted against the temperature. Sigmoidally shaped stability curves – as for DSF assay – are obtained and the inflection point (T_m value) can be determined.

For a measurement, about 10 µL protein sample are filled into a capillary (Prometheus NT.Plex nanoDSF Grade Standard Capillary Chips) and placed on a thermal element inside the measuring instrument (Prometheus NT.48, nanoTemper). The heating program starts with a heating rate of 1.5 K/min from 15 to 95 °C while the fluorescence signal is detected. For analysis, the software PR.ThermControl from nanoTemper is used and the first derivative of the plots is calculated. The inflection points or rather the peak maxima can be determined, and the T_m values read out easily.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method to separate negatively charged SDS-protein complexes by their molecular mass in an electric field. The negative charge results from the denaturation with SDS. Thereby, the denaturation ensures that the protein is linear, without aggregation and with a negligible protein charge. As result the proteins can be separated by their molar mass.[294] A discontinuous PAGE has a stacking and a separation gel. The stacking gel has an acrylamide concentration of 4%, the separation gel of 12%. Both gels are purchased from Invitrogen (NuPAGE 4-12% Bis-Tris Protein Gels, 1 mm, NP0322BOX). Running buffer is a mixture

of MES (50 mM), TRIS (50 mM), SDS (0.1%) and EDTA (1 mM) with a pH value of 7.3 (NuPAGE, MES SDS Running Buffer, NP0002).

For sample preparation, samples with α-amylase or lipase are diluted with running buffer and denatured at 95 °C. Samples containing protease need a precipitation due to the autoproteolysis properties of the enzyme. Protease solution is added to trichloroacetic acid (TCA, 50%). The precipitated protease is centrifuged, the supernatant removed, and the pellet is washed three times with acetone. After evaporation of the solvent, the residue is dissolved in sodium acetate (100 mM, pH=4.6) and further diluted with running buffer.

Gels are fixed into a gel chamber (X-Cell ShureLock, Invitrogen) and bubble-free filled with running buffer. Combs are removed, and the obtained gel pockets are filled with 20 µL sample respectively. Marker is SeeBlue® Plus2 Pre-stained Protein Standard (LC52925) with a range of 3 to 198 kDa. Afterwards, the chamber is closed and connected to a voltage source (200 V, 120 mA). Gel electrophoresis runs about one hour and as a result all proteins are sorted by their size. For further analysis protein staining is used. Therefore, the gel swiveled in a staining solution (0.25 g Coomassie Brilliant Blue R250, 250 mL methanol, 50 mL acetic acid, 200 mL water) for 15 minutes. Then, the gel is swiveled in a decolorizing solution (25 mL methanol, 35 mL acetic acid, 440 mL water) for three to six hours.

High Performance Liquid Chromatography (HPLC)

Chromatography is a separation process based on a mobile phase (eluent) containing the sample that is pumped with high pressure through a stationary phase (surface-active solid material) inside a column. Depending on the strength of interaction between sample and stationary phase, the retention time of the samples varies. Strong interactions lead to a long time on the column and a deferred retention time. At the end of the separation column is a detector – mostly UV – that records the sample divided into fractions. Most common is the use of long-chain hydrocarbon modified silanes as stationary phase. Such reversed phase (RP) columns decrease the retention time of polar samples. Furthermore, the mobile phase can be varied (gradient separation) or remain unchanged (isocratic separation) during elution.[295]

The HPLC system used is from Agilent Technologies and consists of a high-performance degasser (Agilent 1260 Infinity), a binary pump (Agilent 1260 Infinity), an autosampler (Agilent 1260 Infinity), the column (Water, Acquility UPLC Protein BEH C4) and an UV/VIS detector (diode array detector (DAD), Agilent 1260 Infinity). Stationary phase is a reversed phase (RP) column with a C4 ligand. Mobile phase is gradient of solvent A (acetonitrile, 0.1% trifluoroacetic acid (TFA)) and solvent B (water, 0.1%

TFA). Flow rate during separation of samples is 0.5 mL/min. The DAD measures the absorption at 280 nm. For analysis, the software package Chromeleon (Version 7.2, ThermoFisher) is used.

Asymmetrical-Flow Field-Flow Fractionation (AF4)

Field-flow fractionation (FFF) is a chromatography related technique to separate particles. The basis is an external field or a gradient that is perpendicular to the direction of movement of a sample inside a small channel. The generation of the external field enables a distinction between different FFF variants. Centrifugal force of a centrifuge is used for sedimentation FFF, for instance. Asymmetrical-flow field-flow fractionation (AF4) uses a fluid flow and the separation is based on varied diffusion coefficients of differently-sized particles. Using AF4 it is possible to determine the hydrodynamic radius of the fractions through the angular distribution of the multiangle light scattering (MALS). In addition, the molar mass of the fractions in a range of 10^3 to 10^{12} g/mol can be investigated with quasi-elastic light scattering (QELS) by calibration with a standard. Furthermore, the instrument is equipped with an UV (280 nm) and a refractive index (RI) detector.[296]

For the measurements, phosphate buffer (100 mM, pH=10) or TRIS buffer (4 mM, pH=7.2) is used as eluent. Samples with an enzyme concentration of 2 to 4 mg/mL are injected into the fractionation channel. This long channel with a height of 350 µm is equipped with a 10 kDa membrane of regenerated cellulose (Millipore). The separations are carried out at room temperature and start with a focusing/ injection step of three minutes with a focusing flow rate of 3 mL/min and an injection flow rate of 0.2 mL/min. Overall 20 µL sample are injected into the channel. Subsequent, a relaxation time of one minute is maintained. An elution step is performed with a constant cross-flow for 15 minutes at 3 mL/min followed by an exponentially decreasing cross-flow rate from 3 to 0.1 mL/min in ten minutes. The detector flow rate is 0.1 mL/min. Molar masses are determined using MALS and the specific refractive index increment of dn/dc=0.15 for scattering angles from 37.5 to 140°. Zimm formalism with a first-order exponential is used for data fitting. For analysis, the software package Astra 6.1.5.22 (Wyatt Technology Corporation) is used.

Powder X-Ray diffraction (PXRD)

Powder X-ray diffraction (PXRD) is used to determine the crystallinity and the grid structure of a sample (MOF for instance). X-ray is electromagnetic radiation with a wavelength of about 100 pm.[297] It can be generated by firing of high-energy electrons towards a metal surface. By hitting the metal, the electrons are slowed down suddenly and radiation with a continuous spectrum of wavelength

(bremsstrahlung) is released. In addition, electronic excitation results into a discrete spectrum with a wavelength that is characteristic for the used metal ($K_{\alpha 1/2}$ or K_{β} radiation).

Using X-ray diffraction, monochromatic X-ray with a wavelength λ irradiates a sample and the intensity of the scattered light is detected as a function of the reflection angle 2θ to the primary bean. For most incidence angles θ, the wavelength difference of diffracted beams is not an exact multiple n of the wavelength λ and the interference is destructive. Incidence angles with constructive interference fulfill the Bragg condition (equation 2; d: distance of lattice plane):

$$n \cdot \lambda = 2 \cdot d \cdot \sin(\theta) \quad (2)$$

Crystals of a powder sample show all orientations towards the incident beam and intensity maxima are present. Crystal lattice have periodic distances d of parallel lattice planes resulting in characteristic powder X-ray diffractograms.[298]

PXRD measurements are conducted on a Rigaku Miniflex 600 (Bragg-Brentano geometry). X-ray is derived from Cu-K_{α} radiation of a hot cathode (λ=1.54056 Å). All samples for PXRD are dried before measurement between 2° and 50° 2θ, with 4°/min.

Transmission Electron Microscopy (TEM)

Resolution of a transmission electron microscopy (TEM) is appreciably higher than with a light microscope due to the use of short-wave electrons, which are generated by thermal emission of a hot cathode in a high vacuum and focused using electromagnetic lenses. Electron radiations meet the sample and some electrons are scattered due to interactions with sample atoms. Thereby it can be distinguished between elastic and inelastic scattering. In the first case the kinetic energy of the electrons is before and after the collision the same due to the lower mass of the electrons compared to the nucleus. The so-called Rutherford scattering is responsible for the image generation. Inelastic scattering, the second case, occurs if as much energy of the incident electron is transferred onto an electron of the atomic shell that the latter electron is separated from the atom. Inelastic scattering is element-specific and used for energy-dispersive X-ray spectroscopy (EDX). Nevertheless, elastic scattered electrons that leave the sample under the same angles are focused by the objective lens after passing an aperture. The intermediate image is enlarged further using a lens system and displayed finally on a fluorescent screen. In general, the sample must be thin to avoid to much absorption of the electrons and an electrostatic charge. High vacuum around the sample is also necessary in order to impede interactions of the electrons with gas particles.[299-300]

TEM samples are prepared by dropping suspensions onto copper grids with lacey carbon support. The grids are air dried for one day. TEM imaging is performed by the Philips Tecnai 12 with an accelerating voltage of 120 kV.

4.2.3. Enzyme Performance Tests

In order to identify the need of enzyme stabilization and to test the enzyme stabilization strategies used, enzyme performance tests have been done. The first tests in lab are enzyme storage tests in liquid standard detergent formulation to track enzyme stability over time. Identified lead candidates are further tested in washing simulation performance tests against stained textiles.

Storage Tests in Standard Detergent Formulations

In the storage tests, the enzyme concentration in liquid detergents is different for protease, α-amylase and lipase. Protease is the enzyme with the highest concentration (0.9 mg/mL), followed by α-amylase with 0.2 mg/mL. Lipase has a concentration of 0.05 mg/mL. Pure and stabilized enzymes are diluted to those concentrations for the storage tests. The used standard detergent formulation contains respective one LAS, FAES and FAEO. A builder is not present, and the pH value is adjusted to 8.5 using sodium hydroxide. At the time of enzyme addition to the detergent time measurement starts, and the first sample is taken. Further samples are taken after about 4 h, 1 d, 2 d, 7 d, 14 d, 21 d and 28 d. The solutions are stored at 22 or 37 °C. As a reference the pure enzyme in water is measured at each storage test additionally.

For testing the stability of α-amylase and especially lipase against protease, the enzymes with their respective concentrations are mixed in water and stored like described before.

Washing Performance Tests

For washing tests, 200 mL standard detergent formulation is added to 48 mL hard water. The water hardness is 2.5 mM (Ca/Mg/HCO_3 4:1:8). 2 mL enzyme solution is added to that mixture. A total of 250 mL washing solution with 10 g cotton fabrics as ballast and 10 g stained textile as well as 20 steel balls are filled into a washing container. The container is placed inside the Launder-O-meter (LOM) and the washing process is started. It is washed for 30 minutes at 25 or 40 °C. L*a*b values of the stained textile are determined photometrically before and after washing.

4.2.4. Procedures Used for Enzyme Stabilization

Small Molecules

Preparing of Solutions

The order of mixture is always the same: water, standard detergent formulation, additive, enzyme. Enzyme concentration for nanoDSC experiments is 2 mg/mL with a standard detergent formulation that is diluted to 50%.

Concentrations Decomposed Esters

Acetylcholine: 1wt.-% choline, 0.4wt.-% acetic acid.

Citric acid choline ester: 0.5wt.-% choline, 0.4 wt.-%citric acid.

Triethyl citrate: 0.3wt.-% ethanol, 0.4wt.-% citric acid.

Acetyl triethyl citrate: 0.2wt.-% ethanol, 0.3wt.-% citric acid, 0.1wt.-% acetic acid.

Enzyme-Polymer Conjugates

Oxidation of Polysaccharides

The polysaccharides tested (CMC and maltodextrin) are oxidized according to YI ET AL. by using sodium hypochlorite and sodium bromide. Sodium hypochlorite is slowly added to the polysaccharides dissolved in water (pH=5-6). Temperature (between 5 and 22 °C) and pH value are held constant.[301] Oxidized polysaccharides are purified by dialysis.

Modification of Enzymes with mPEG- or Polysaccharide-Aldehydes

The enzyme is dissolved in phosphate buffer (0.1 M, pH = 9) and the appropriate polysaccharide-aldehyde (CMC or maltodextrin) is added with a molar excess of 50 with respect to the enzyme. After 2 h of stirring at 22 °C, sodium cyanoborohydride is added with a tenfold molar excess with respect to the polymer. Stirring continued for one to three days at room temperature. The enzyme conjugate is purified by dialysis and further lyophilized.

Modification of Enzymes with mPEG-NHS

The enzyme is dissolved in phosphate buffer (0.1 M, pH = 9) and mPEG-NHS with a molar excess of about 50 in respect to the enzyme is added to the solution. The mixture is stirred at 22 °C for one day, before the enzyme-conjugate is purified by dialysis and then lyophilized.

Mesoporous Silica Nanoparticles

Synthesis of TEOS-OTMS Particles (by Adnan Khalil)

According to KALANTARI ET AL.,[209] 68 mg of the base-catalyst triethanolamine (TEA) are dissolved in 25 mL water at 80 °C under intensive stirring. Afterwards, 380 mg cetyltrimethylammonium bromide (CTAB) and 83 mg sodium salicylate are added as structure-directing agents. The solution is stirred for 1 h, before 3.8 mL tetraethyl orthosilicate (TEOS) and 0.38 mL octadecyltrimethoxysilane (OTMS) are added. The resulting mixture is stirred for 2 h. Then, the as-synthesized sample is collected by centrifugation and washed with ethanol to remove residual reactants. Template is removed by extraction with a 0.01 M solution of hydrochloric acid in ethanol for three days.
For calcination, parts of the synthesized material are treated for 6 h at 550 °C.

Synthesis of BTSE-OTMS Particles (by Adnan Khalil)

68 mg of the base-catalyst triethanolamine (TEA) are dissolved in 25 mL water at 80 °C under intensive stirring. Afterwards, 380 mg cetyltrimethylammonium bromide (CTAB) and 83 mg sodium salicylate are added as structure-directing agents. The solution is stirred for 1 h, before 3.17 mL 1,2-bis(triethoxysilyl)ethane (BTSE) and 0.38 mL octadecyltrimethoxysilane (OTMS) are added. The resulting mixture is stirred for 2 h. Then, the as-synthesized sample is collected by centrifugation and washed with ethanol to remove residual reactants. Template is removed by extraction with a 0.01 M solution of hydrochloric acid in ethanol for three days.
For calcination, parts of the synthesized material are treated for 6 h at 550 °C.

Synthesis of SBA-15 Particles (by Adnan Khalil)

4.0 g of Pluronic P123 are dissolved in 120 g hydrochloric acid (2 M) and 30 g water. The mixture is stirred vigorously for several hours at 35 °C before the template is dissolved completely. In addition, 8.5 g TEOS are added and the solution is stirred for 24 h at 35 °C. Afterwards, the solution is transferred into a polypropylene bottle, sealed and aged at 90 °C for 24 h. By filtration a colorless precipitate is gained. The precipitate is washed with water, dried in vacuum at 40 °C over-night and finally calcinated at 550 °C for 6 h.

Functionalization of SBA Particles with 3-aminopropyltrimethoxysilane (APTMS) (by Adnan Khalil)

0.5 g of the synthesized SBA-15 particles are suspended in anhydrous toluene, before 0.63 mL APTMS are added under nitrogen flow. Afterwards, the suspension is stirred for 20 h at 80 °C. The cooled solution is filtrated and the precipitate washed with toluene and dried in vacuum at 40 °C over night.

Sample Preparation for Attenuated Total Reflection Infrared Spectroscopy (ATR-IR) (by Adnan Khalil)

TEOS-OTMS and BTSE-OTMS nanoparticles are dried in vacuum at 40 °C for 12 h before measurement. ATR-IR measurements are performed using a Perkin Elmer Instrument One Spectrum FT-IR Spectrometer equipped with a Universal ATR Polarization Accessory (Waltham, USA). The spectra are averaged from at least three spots and normalized to the Si-0-Si band at 1085 cm^{-1}. Spectra are recorded using the Spectrum Software (Version 10.5.4.738, PerkinElmer Inc., USA) between 4000 and 500 cm^{-1} with a resolution of 4 cm^{-1}. A background correction is applied automatically.

Sample Preparation for Scanning Electron Microscope (SEM) (by Adnan Khalil)

Mesoporous silica nanoparticles are dispersed in ethanol with a concentration of 0.1% using an ultrasonic bath. 2 µL of the dispersion are placed on a silicon wafer. Subsequently, the sample on the wafer is sputtered with a 5 to 6 nm thick Pt/Pd layer. SEM imaging is performed by HREM Philips XL30 FEG with an accelerating voltage of 30 kV.

Testing of pH Stability (by Adnan Khalil)

Mesoporous silica nanoparticles are dispersed in aqueous solutions with pH values ranging between 10 and 11. Samples are taken after 2, 4, 6, 8 and 24 h incubation. After centrifugation for 5 minutes at 4500 rpm, the pellets are dried in vacuum at 40 °C for 12 h. Dried samples are measured by ATR-IR.

Encapsulation of Enzymes in Mesoporous Silica Nanoparticles

An enzyme stock solution with a final concentration of 1 mg/mL is prepared using water or phosphate buffer (100 mM, pH=3 to 8). Using the same buffer, silica nanoparticles are dispersed. Subsequently, 1 mL enzyme stock solution is added to 0.5 mL nanoparticle solution and the dispersion is stirred for 24 hours. Afterwards, the reaction solutions are centrifuged for five minutes at 6700 x g. The supernatant is removed and used for enzyme concentration assay (BCA assay, chapter 4.2.2.). Redispersed pellet is washed three times with water, before enzyme activity is determined for pellet and supernatant using an enzyme specific assay (chapter 4.2.1.).

Metal-Organic Frameworks

Preparing Precursor Solutions

In separated solutions, the MOF precursors are dissolved in water. Thereby, the pH value of the solutions is adjusted to 8.5, except for 2,5-dihydrodyterephthalic acid and terephthalic acid. In the two latter cases, pH is adjusted around 9 due to their solubility in water. In table 4-6 the molar concentrations of precursor solutions are listed.

Table 4-6: Molar concentrations of precursor solutions.

Precursor	Concentration [M]	MOF
2-methylimidazole	2	ZIF-8
Zinc acetate dihydrate	0.4	ZIF-8, MOF-74
2,5-dihydroxyterephthalic acid	0.3	MOF-74, MIL-53-X
Terephthalic acid	0.1	MIL-53
Aluminum nitrate nonahydrate	1.3	MIL-53, MIL-53-X

Synthesis of Metal-Organic Frameworks

The enzyme used is diluted using water to a stock solution with an enzyme concentration of 5 mg/mL. In general, the necessary amount of water is filled into a glass vessel and a defined quantity of enzyme stock solution is added. Subsequently, one by one are added the organic linker and the metal salt solution under continuous stirring. Final enzyme concentration is between 0.25 and 0.125 mg/mL and the concentration range of the precursors is shown in table 4-7. Aqueous solutions containing enzyme, organic linker and metal salt are stirred continuously for 24 h at room temperature. Afterwards, the reaction solutions are centrifuged for five minutes at 6700 x g. The supernatant is removed and used for enzyme concentrations assays: BCA and UV-assay (chapter 4.2.2.). Redispersed pellet is washed three times with water, before enzyme activity is determined for pellet and supernatant using an enzyme specific assay (chapter 4.2.1.).

Table 4-7: Final precursor concentrations in the MOFs formed.

Precursor	Concentration [mM]
2-methylimidazole	25 – 800
Zinc acetate dihydrate	0.625 – 30 (ZIF-8) and 7 – 58.6 (MOF-74)
2,5-dihydroxyterephthalic acid	3 – 25 (MOF-74 and MIL-53-X)
Terephthalic acid	9 – 44 (MIL-53)
Aluminum nitrate nonahydrate	17 – 67 (MIL-53 and MIL-53-X)

Characterization of Metal-Organic Frameworks

In order to perform PXRD and TEM measurements, pellets of centrifuged MOFs are redispersed with methanol and dried by gently compressing. Afterwards, the dry pellets are transferred to appropriate slides.

5. Results and Discussion

This chapter is divided into several parts. Besides the four strategies to stabilize enzymes, reference experiments where the enzymes stability in a liquid standard detergent formulation amongst other conditions is tested are shown firstly.

5.1. Reference Experiments

In this chapter the need of stabilization strategies for enzymes in liquid detergent formulations is outlined. Therefore, the three enzymes of interest, namely a protease, α-amylase and lipase, are characterized briefly regarding pH and proteolysis stability as well as stability in a liquid standard detergent formulation. Enzyme activity in storage tests and conformational stability serve as yardstick for enzyme stability. Concerning the determination of conformational stability, three different techniques are tested and compared. Finally, a correlation between conformational stability and residual enzyme activity is explored using the Gibbs-Helmholtz equation.

5.1.1. pH Stability

In first instance, protease, α-amylase and lipase are analyzed regarding their pH stability. Since an alkaline pH value (about 8.5) in liquid detergent formulations is present, the effect of pH on enzymatic stability is investigated. Enzyme stability is determined measuring enzyme activity after 6 days storage in buffer solutions with corresponding pH values and of the conformational stability by nanoDSC, DSF assay and nanoDSF. A pH range from 3 to 11 is investigated and the results obtained measuring enzyme activity are shown in figure 5-1 A. Using the same phosphate buffer solutions, the conformational stability of the three enzymes is determined (figure 5-1 B to D). In figure 5-1, the thermal transition values (T_m) are plotted against the pH value. Complete thermograms of the nanoDSC, DSF and nanoDSF measurements are shown in the appendix (chapter 11.1., figure 11-1 (nanoDSC), figure 11-3 (nanoDSF), figure 11-5 (DSF assay)).

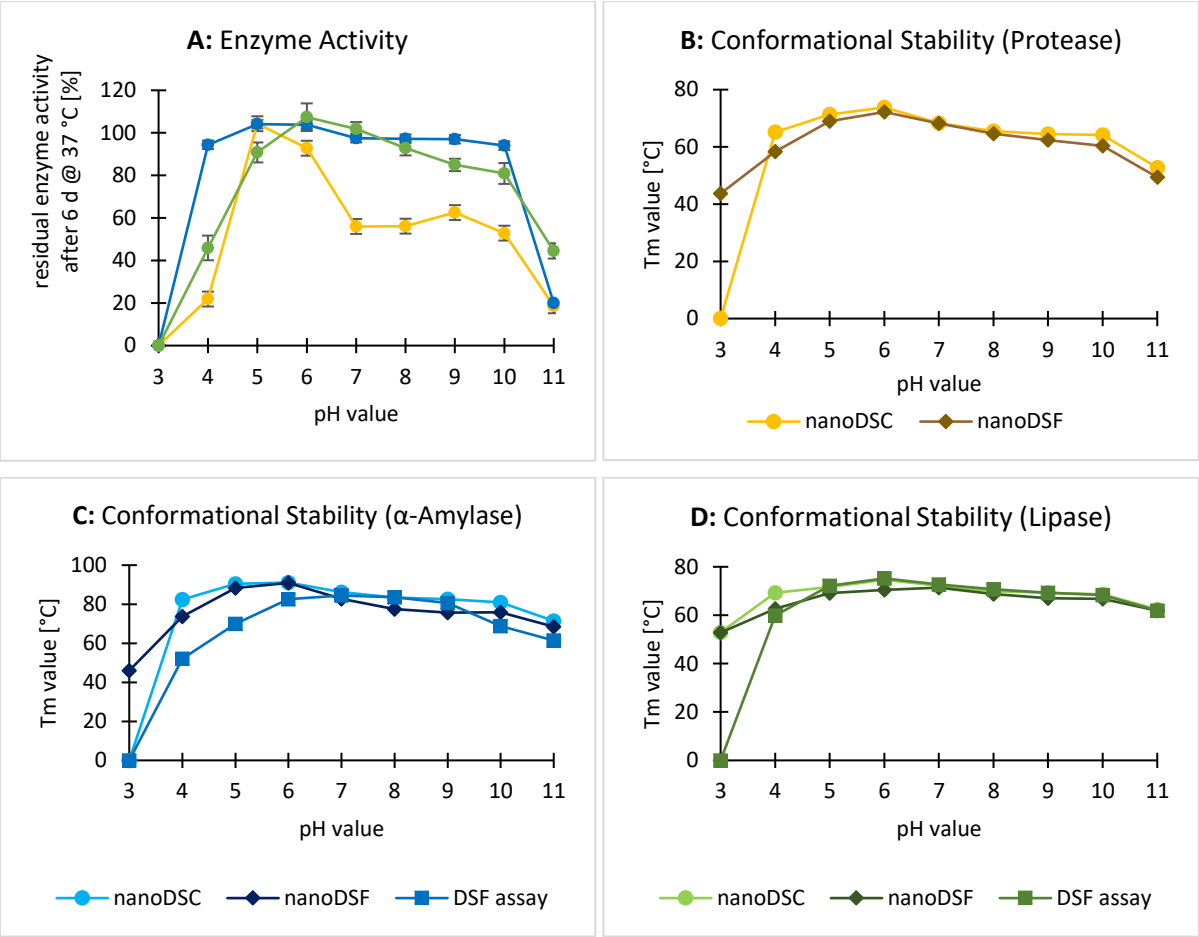


Figure 5-1: Effect of storage pH on enzyme activity after 6 days at 37 °C (A) ● protease ● α-amylase ● lipase. Conformational stabilities determined by ● nanoDSC ● nanoDSF ● DSF assay are plotted for protease (B), α-amylase (C) and lipase (D). Connecting lines are only guide to the eye. The average error for conformational stability experiments in aqueous solutions is 0.5 K. For original data see appendix chapter 11.1., figure 11-1 (nanoDSC), figure 11-3 (nanoDSF), figure 11-5 (DSF assay).

Figure 5-1 shows a pH stability of α-amylase between pH 5 and 10. The results in enzyme activity (figure 5-1 A) are confirmed by conformational measurements (figure 5-1 C). At pH values higher than 10 the enzyme activity is significantly reduced after 6 days of storage at 37 °C. This can be explained by the composition of the active site where Glu261 serves as a proton donor and Asp231 as a nucleophile.[302] In literature the pH optimum is determined in the range of pH 7 to 9.[43]

Protease is stable between pH 5 and 6 with the highest residual activity and T_m value at pH 5 and 6. At pH values between 7 and 10 the protease is relatively stable, whereas the enzyme is unstable at more alkaline conditions. Storage test (figure 5-1 A) and conformational stabilities (figure 5-1 B) demonstrate similar results for protease stability. Enzyme activity (figure 5-1 A) and conformational stability (figure 5-1 D) confirm that lipase is stable between pH 5 and 9. The highest residual activity and T_m value are obtained at pH 6. The pH stability of protease and lipase can be explained by the composition of the active site as well. Both enzymes possess a Ser-His-Asp catalytic triad (chapter 2.2.)

and the overall pK_a value of those residues indicates the preference for an alkaline pH.[41] In literature a pH stability between 4 and 11 for protease and lipase is reported.[303-304]

For the determination of the conformational stability, three different methods have been used: differential scanning fluorimetry (DSF), nano differential scanning fluorimetry (nanoDSF) and nano differential scanning calorimetry (nanoDSC). All three methods determine a thermal transition point (T_m value) at which half of the protein present is denatured, while the other half possess its native conformation, is determined. Differences between corresponding T_m values are relatively small and the methods show the same trends regarding the enzyme stability in buffer solutions at various pH values (figure 5-1 B to D). Major differences between the three techniques occur at lower pH values. It should be noted that the conformational stability of protease cannot be measured using the DSF assay under the tested conditions. The autoproteolysis of this enzyme disturbs the interactions between enzyme and dye. As a result, only a small amount of dye is able to bind to the hydrophobic enzyme parts and only a weak fluorescence signal, which is not evaluable, can be detected. One possibility to bypass this issue is to inactivate the protease irreversible to stop the autoproteolysis. However, the results obtained by the three different methods are not comparable anymore, if an inhibitor is used for the DSF assay.

The differences regarding the T_m values between the three methods are even greater if the thermal stability is measured in a liquid standard detergent formulation. It is difficult to measure an optical signal within a standard liquid detergent formulation. The ingredients present, especially micelles formed by surfactants, can interact with dye of a DSF assay resulting in a manipulated fluorescence signal. Using nanoDSF, the intrinsic fluorescence can be quenched by the detergent formulation. Nevertheless, the results of nanoDSF measurements in an SDF and in water are shown in the appendix (chapter 11.1., figure 11-4). Differential scanning calorimetry is the most reliable method in detergent formulations. This method dispenses the usage of a dye and is independent of enzyme structure properties (number of tryptophan or tyrosine). Draw-backs against the two other methods are the time-consuming measurements (ten samples per day versus 96 (DSF) or 48 (nanoDSF) in two hours) and the relatively large amounts of protein that are necessary (1 g per sample versus about 4 µg). Nonetheless, the following experiments in the present thesis regarding thermal stability are conducted with the nanoDSC.

5.1.2. Enzyme Stability in a Liquid Standard Detergent Formulation

The three detergent enzymes of interest – protease, α-amylase and lipase – are added to a standard detergent formulation (SDF) under typical concentrations and ratios. Background of this storage test is to identify the need of enzyme stabilization. Again, the stability of the enzymes is determined by

measuring the enzyme activity and the thermal stability (figure 5-2). For the enzyme activity measurements, the samples are stored at two different temperatures: 22 and 37 °C. Conformational stabilities are investigated using nanoDSF, DSF assay and nanoDSF. It should be noted that the concentrations of enzyme and detergent for these measurements differ from the standard concentrations. The standard detergent formulation is diluted to 50% because of the viscosity limit of the sample needle. In addition, the enzyme concentration is increased about twofold (protease), tenfold (α-amylase) and fortyfold (lipase) due to the detection limit of the instrument. Despite the differences the results obtained are comparable and show similar tendencies (figure 5-2). Thermal stability data (T_m values) in figure 5-2 D originate from nanoDSC experiment. Corresponding thermograms of nanoDSC are shown in the appendix (chapter 11.1-, figure 11-2).

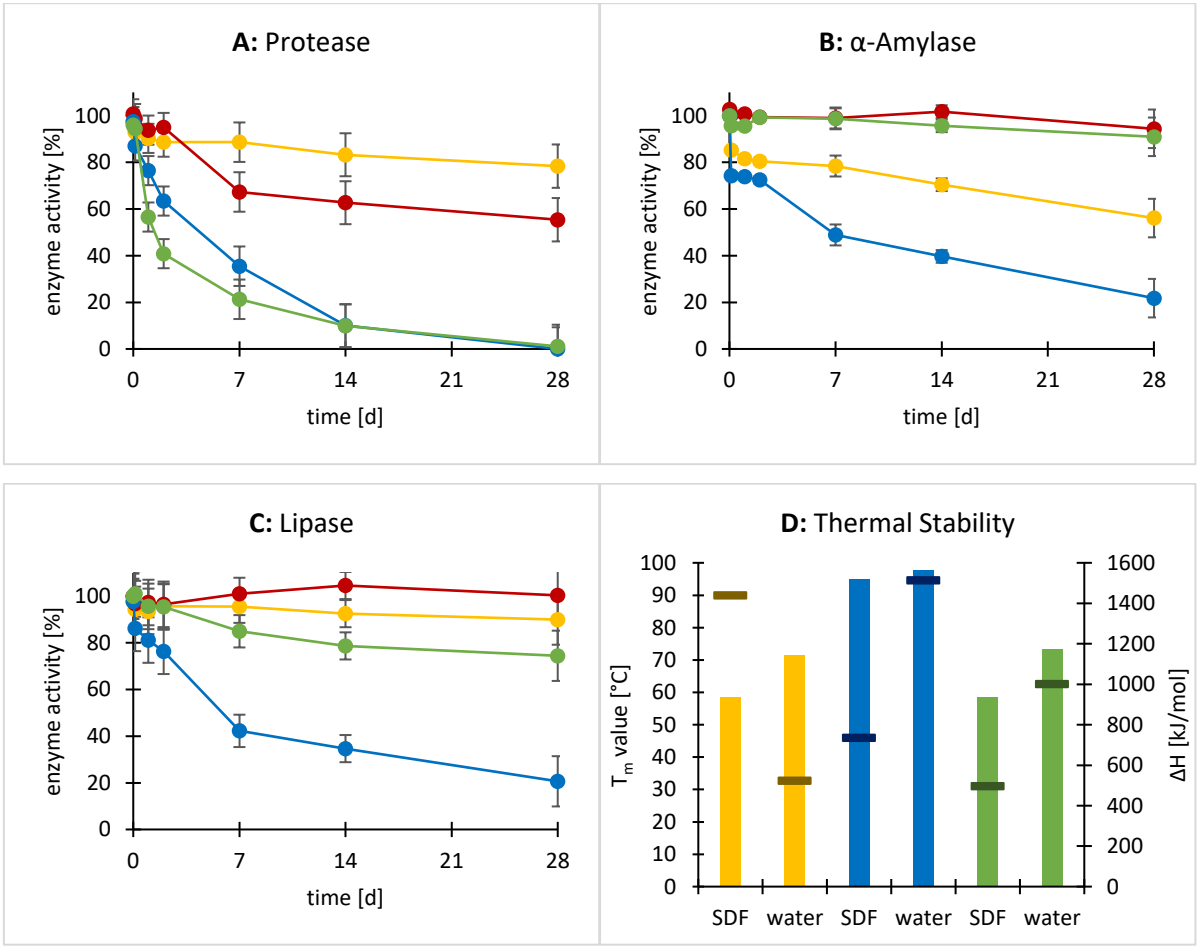


Figure 5-2: Enzyme stability in a standard detergent formulation (SDF) and in water. The stability is determined by measurement of the enzyme activity in storage tests and of the thermal stability with the nanoDSC. A: storage test with protease; B: storage test with α-amylase; C: storage test with lipase ● Standard Detergent Formulation (22 °C) ● Water (22 °C) ● Standard Detergent Formulation (37 °C) ● Water (37 °C). Connecting lines are only guide to the eye.
D: thermal stability of all three enzymes. T_m values are displayed as bars (first Y axis), calorimetric enthalpy (ΔH_{cal}) as dark lines (second Y axis). ● protease ● α-amylase ● lipase. The average error for nanoDSC experiments in aqueous solutions is 0.5 K, for detergents 1.5 K. For original data see appendix chapter 11.1., figure 11-2.

Lipase and α-amylase are both stable in water and the enzyme activity remains almost completely at 100% independent from the storage temperature (figure 5-2 B and C). The SDF decreases the activity of lipase to 90% and of α-amylase to about 60% after 28 days at 22 °C. At a storage temperature of 37 °C the activity after four weeks is reduced to 20% in both cases. The loss of enzyme activity is attributed to the presence of surfactants, especially of LAS in the SDF. It is known from literature that LAS are highly detrimental for enzymes.[305] Interestingly, the denaturing effect of LAS is shortened if a second anionic surfactant built up as FAES is present.[54, 306] Experiments regarding thermal stability underline the detrimental effect of SDF on α-amylase and lipase. In both cases the T_m value is shifted to lower temperatures compared to water (lipase: 15 K; α-amylase: 3 K) which indicates a conformational enzyme destabilization. Additionally, the peak area is significantly reduced which indicates a loss of folded enzyme (figure 5-2 D).

Compared with the other two enzymes, lipase and α-amylase, the protease activity is decreased most in the storage tests (figure 5-2 A). After four weeks storage at 37 °C no enzyme activity can be measured in water or SDF. At 22 °C the protease activity after 28 days is decreased to 55% (water) and about 80% (SDF) respectively. It is notable that protease seems to retain more enzyme activity in SDF than in water, which is related to the autoproteolysis of the enzyme. Proteases catalyze the cleavage of peptide bonds and the own peptide bonds are not spared which results in a self-digestion.[2] Autoproteolysis is responsible for the activity loss in water and is less pronounced if the amount of water is reduced like in a standard detergent formulation. For hydrolysis a water molecule is essential.[36] In an SDF, the reduction of activity is caused by a lowered autoproteolysis combined with the presence of surfactants. In the nanoDSC, the autoproteolysis is expressed by a broad flat peak as shown for protease in water. In contrast, the peak obtained for protease in SDF is sharper und higher due to a reduced autoproteolysis. The location of the peak maximum, T_m value, is only little influenced by the autoproteolysis so that the T_m value in SDF is strongly shifted (13 K) to lower temperatures (figure 5-2 D).

5.1.3. Stability of Protease, α-Amylase and Lipase in a Mixture

Additionally, the three enzymes tested are stored together in one solution just as in a real detergent. Here, proteolytic properties of protease are investigated in an SDF at 37 °C and in water at 22 °C. The results of enzyme activity measurements are shown in figure 5-3.

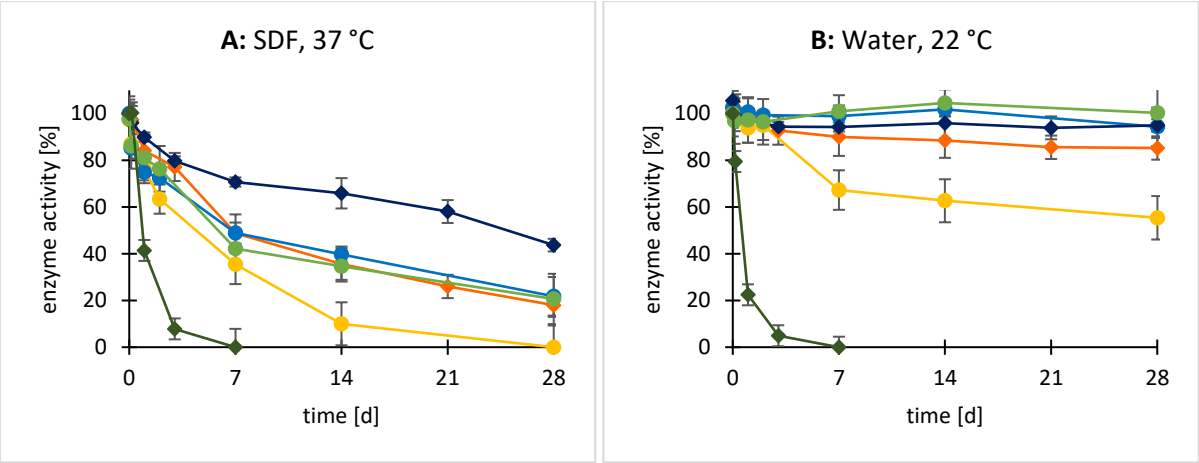


Figure 5-3: Stability of an enzyme mixture (protease, α-amylase and lipase) in a standard detergent formulation at 37 °C (A) and in water at 22 °C (B). ● pure protease ◆ protease mixture ● α-amylase ◆ α-amylase mixture ● lipase ◆ lipase mixture. Connecting lines are only guide to the eye.

In figure 5-3 activities of the enzyme mixtures and of single enzyme storage are plotted. The presence of protease and lipase has a positive effect on the α-amylase activity in an SDF. As a result, the stabilities observed are significantly increased as compared to α-amylase stored separately in an SDF. In water, α-amylase is stable in a mixture and stored separately (figure 5-3). On the contrary, lipase is degraded by protease. Thus, after one week no lipase activity is left within an SDF and water. Pure lipase shows an increased stability than in the mixture. The presence of a substrate (lipase) stabilizes protease. Consequently, an increased storage stability is observed in an SDF and in water. In general, proteases digest preferable proteins with a flexible loop region.[2] From the results it can be assumed that the lipase tested bears a flexible loop region, contrary to α-amylase that is not affected by protease.

5.1.4. Correlation between Enzyme Activity and Thermal Stability

In the previous chapters the enzyme stability is determined by enzyme activity and conformational stability measurements. This section deals with the development of a method to correlate both techniques. Therefore, all following factors obtained by nanoDSC measurements are considered and cumulated in one value, Gibbs free energy (ΔG) using the Gibbs-Helmholtz equation (equation 1, chapter 4.2.2.): area under the peak (ΔH), peak position (T_m), the molar heat capacity (C_p) and storage temperature (T). Residual enzyme activities after storage for six days at 37 °C are plotted against the calculated values of Gibbs free energy ΔG in figure 5-4. In this way, not only the thermal transition values (T_m) are considered but also the other values obtained by nanoDSC measurements. Accordingly, linear correlations between conformational stability and residual enzyme activity are obtained

(figure 5-4). This linear correlation can be used to save time-consuming storage tests. For instance, LUND ET AL. correlate T_m values and residual enzyme activities qualitatively.[2] Thus, it is possible to make a statement of the enzyme stability at certain storage conditions. Concrete propositions on residual enzyme activities cannot be given. In the present thesis, a quantitative statement of the correlation between conformational stability and enzyme activity is made (figure 5-4).

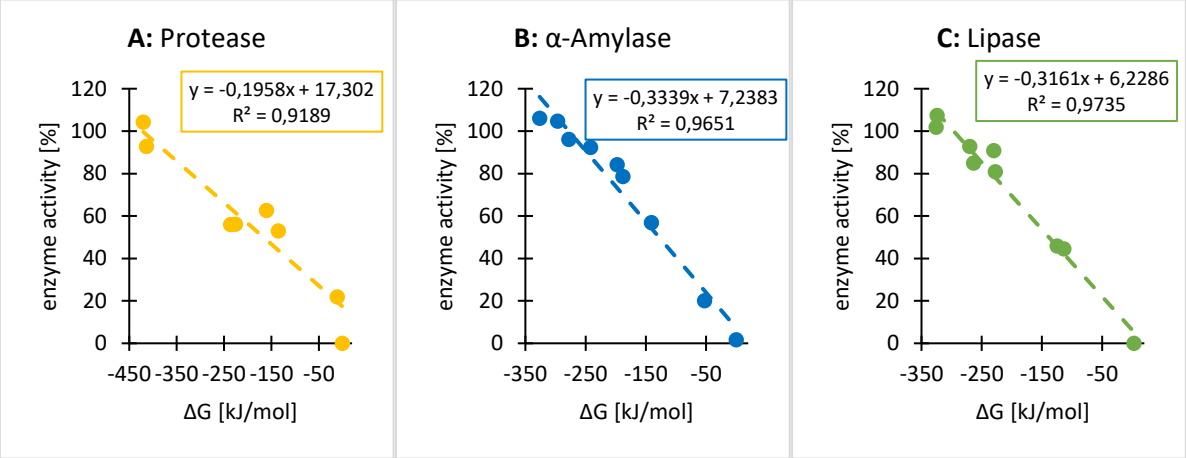


Figure 5-4: Correlation between enzyme activity measured by storage tests and Gibbs free energy calculated from nanoDSC measurements.

For all three enzymes, a linear correlation between thermal stability (ΔG) and residual enzyme activity has been identified. In this context, lipase and α-amylase show a quite similar trend line (figure 5-4 B+C), whereas protease shows a different behavior with a reduced slope of the balance line (figure 5-4 A). The reason for this behavior can be autoproteolysis that occurs only in the case of protease but not in case of lipase and α-amylase. Based on the linear correlation of figure 5-4, it is possible to predict the residual enzyme activity in a storage test by conducting a single nanoDSC measurement. For instance, lipase is stored in the presence of the chelating agents EDTA, HEDP and citrate as wells as the two surfactants Lutensit ALBN (LAS) and Lutensol AO7 (FAEO). Before lipase activity is measured after six days storage at 37 °C, the solutions are analyzed regarding their thermal stability. Using the Gibbs-Helmholtz equation the Gibbs free energy is calculated. Accordingly, the ΔG value obtained by nanoDSC is inserted into the trendline equation for lipase shown in figure 5-4 C. In this way a calculated residual enzyme activity after six days of storage is obtained. Finally, the calculated value is compared with the measured enzyme activity within the storage test. In figure 5-5 A the measured enzyme activities are added into the diagram showing the correlation between conformational stability and enzyme activity. Measured and calculated enzyme activities are compared in figure 5-5 B.

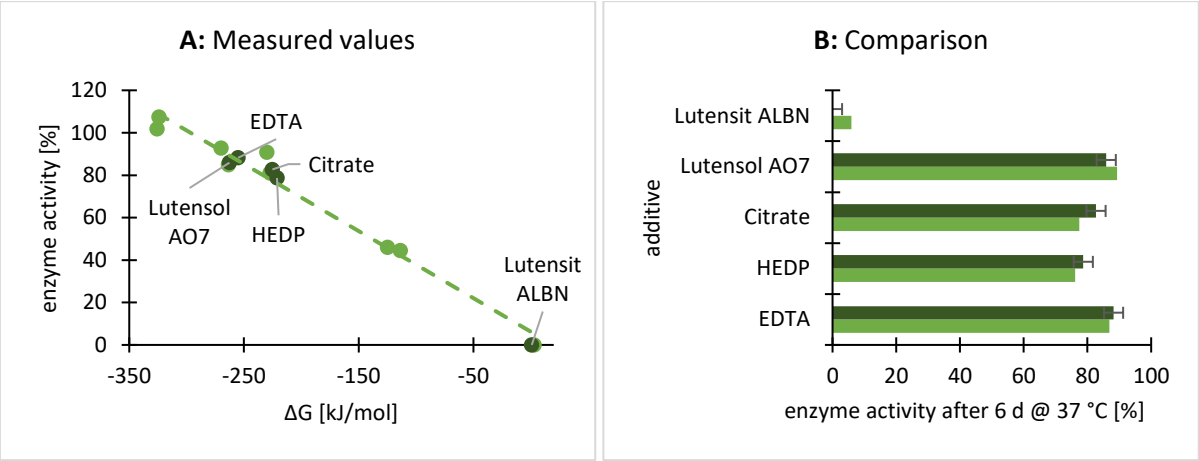


Figure 5-5: Comparison of measured and calculated enzyme. A: Correlation and measured samples. B: Measured and calculated residual enzyme activities of the samples. ● measured enzyme activity ● calculated enzyme activity.

As can be seen from the figure above (figure 5-5), calculated and measured residual lipase activities are quite similar and existing differences are within the scope of the total enzyme activity assay error. It seems that one nanoDSC measurement can replace a time-consuming storage test if an appropriate calibration exists and the surfactant content is below 6% as for Lutensit ALBN and Lutensol AO7. Higher surfactant concentrations – as present in liquid detergent formulation – are not in the calibration mode, so that the correlation between conformational stability and enzyme activity deviates from ideal behavior and storage tests are indispensable. To my knowledge this is the first description of a linear correlation between Gibbs free energy and enzyme activity.

Chapter 5.1. identifies most detrimental conditions for enzymes to maintain enzyme activity as well as conformational stability and finally points out the need of methods to enhance the stability of enzymes. Even if protease, α-amylase and lipase maintain their enzymatic activity and conformational stability at the pH value (8.5) of a buffer solution (chapter 5.1.1.), the enzymes are very sensitive to the surfactants present in detergent formulations. This becomes evident by significantly decreased enzyme activities in storage tests and by reduced thermal stabilities (chapter 5.1.2.). Additionally, lipase and protease are susceptible to proteolytic degradation (chapter 5.1.3.). In order to get an enzyme which is capable to perform in a liquid detergent, stabilization strategies are required. The enzymes have to be protected against the surfactants and against proteolytic degradation. Table 5-1 condenses stabilization needs of each enzyme. Experiments and results regarding chelating agents like ethylenediaminetetraacetic acid (EDTA), 1-hydroxyethane 1,1-diphosphonic acid (HEDP) or citrate are shown in the appendix (chapter 11.1., figure 11-7). Own experiments and literature accord that α-amylase is highly and protease is sensitive to chelating agents due to calcium ions present at both

enzymes. Chelating agents complex those calcium ions that are indispensably for conformation and enzyme activity. In contrast, lipase is stable in presence of even high concentrations of chelating agents.[2]

The conformational stability of the enzymes is determined by three different methods: DSF, nanoDSF and nanoDSC. Experiments within a liquid standard detergent formulation have identified nanoDSC as most suitable for further experiments.

By comparing the results obtained by differential scanning calorimetry and storage tests, a good correlation between both characterization techniques has been detected. This correlation enables a reduction of time-consuming long-term storage tests by one nanoDSC measurement and calculation of the Gibbs free Energy afterwards.

Table 5-1: Summary of vulnerabilities of protease, α-amylase and lipase. Surfactants, proteolysis and pH value are classified regarding the previous chapters. Results regarding chelating agents are not shown here. Color code: green-resistant; red-sensitive; orange-less sensitive.

Enzyme	pH value	Surfactants	Proteolysis	Chelating Agents
Protease				
Amylase				
Lipase				

To achieve an improvement in stability, the enzymes have to be separated from surfactants and protease. Alternatively, the proteolytic activity of protease can be inhibited, or the lipase modified in a manner that it is a poor substrate for protease. In the following chapters several strategies are presented.

Experimental work work regarding enzyme stability is implemented with the collaboration of Olga Pinneker and Annkathrin Morweiser (lab technicians, assistance in conformational stability measurements) and me (remaining work). Parts of the work are published in Kübelbeck, S.; Mikhael, J. *et al.*, Immobilization of α-amylase in polyelectrolyte complexes, *J. App. Polym. Sci.*, **2017**, *134*, 45036 and Kübelbeck, S.; Mikhael, J. *et al.*, Enzyme-Polymer Conjugates to enhance enzyme shelf life in a liquid detergent formulation, *Macromol. Biosci.*, **2018**, *18*, 1800095.

5.2. Small Molecules

In this chapter small molecules are used to increase the stability of enzymes, thereby it is mainly focused on lipase. No stabilizer on the laundry detergent market addresses directly lipase. As mentioned in chapter 2.4. the research in this area focusses on the inhibition of protease using small molecules like 4-FPBA. In figure 5-6 the general approach beginning with the identification of possible stabilizers is displayed.

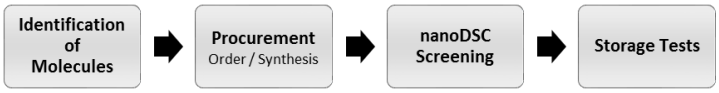


Figure 5-6: General workstream for enzyme stabilization using small molecules.

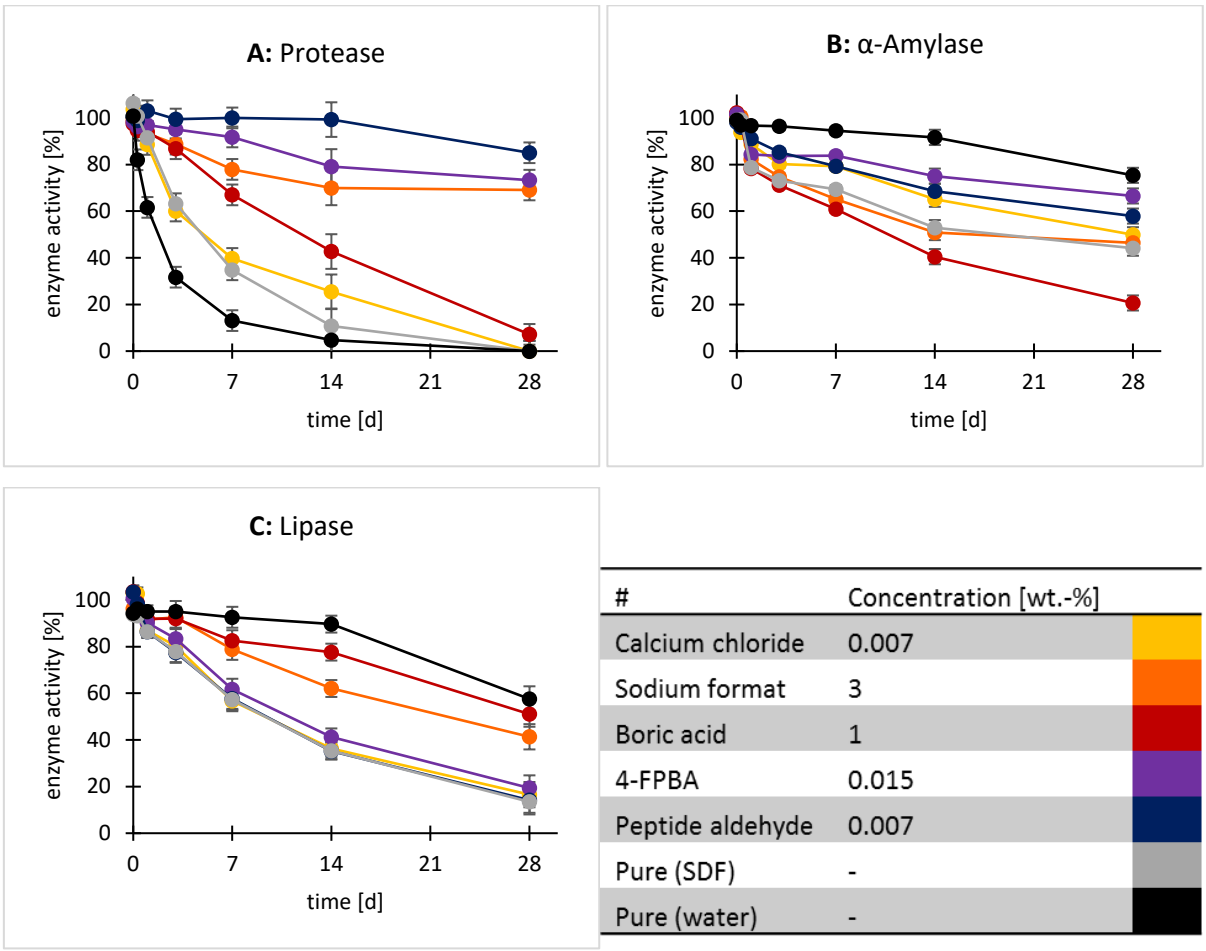
Basis for the selection of small molecules as possible lipase stabilizers is the idea that related enzymes show related affinities. Acetylcholine is the substrate of AChE. Therefore, it is assumed that acetylcholine interacts with the related enzyme lipase as stabilizer or substrate as well. Furthermore, the acetylcholine derivates are tested to determine which parts of the small molecule – for example the positive quaternary amine – are necessary for the effect on lipase. Following this concept, four different small molecules are selected for enzyme studies: acetylcholine (ACh), citric acid choline ester (CACE), triethyl citrate (TEC) and acetyl triethyl citrate (ATEC) (figure 2-11). ACh and TEC are purchased, whereas CACE and ATEC are produced by the lab of Stephan Hüffer.

It should be mentioned that the stabilization strategy described in this chapter is part of a currently running BASF project which aims for a fast market launch. Therefore, parts of the project are strictly confidential and not elaborated further in the present thesis. Nevertheless, in the following chapters the results of concentration screening as well as of storage tests are discussed. Beside the testing of new stabilizers in liquid detergents, the established small molecules are trialed in enzyme storage tests as well as measurements regarding the thermal stability using nanoDSC.

5.2.1. Testing of Established Small Molecule Enzyme Stabilizers in Liquid Detergents

In the first place, established small molecules for enzyme stabilization in liquid detergents, listed in table 2-2, are tested. Therefore, calcium chloride, sodium format, boric acid, 4-FPBA and a peptide aldehyde are added to solutions of protease, α-amylase and lipase in a standard detergent formulation. The exact structure of the peptide aldehyde will not be named due to a running BASF project. Concentrations used in the storage tests comply with the real concentrations in liquid detergents. Whereas the enzyme and detergent concentration in measurements regarding thermal

stability differ as mentioned in chapter 5.1.2. Results of the storage test with protease, α-amylase and lipase at 37 °C are shown in figure 5-7.



#	Concentration [wt.-%]
Calcium chloride	0.007
Sodium format	3
Boric acid	1
4-FPBA	0.015
Peptide aldehyde	0.007
Pure (SDF)	-
Pure (water)	-

Figure 5-7: Enzyme stability in a standard detergent formulation (SDF) with various additives. The stability is determined by measurement of the enzyme activity in storage tests. A: storage test with protease; B: storage test with α-amylase; C: storage test with lipase. Connecting lines are only guide to the eye.

The addition of calcium chloride shows no or only a weak stabilizing effect for all three enzymes tested (figure 5-7). In addition, a precipitation of the surfactants inside the standard detergent formulation resulting in a turbid solution, is observed. Using sodium format as an additive, protease and lipase show an increased stability. In case of stabilization using protease, a residual enzyme activity of about 70% can be measured after four weeks storage, whereas the pure protease shows no activity anymore (figure 5-7 A). For lipase a weaker influence can be observed, so that 40% towards 10% of the enzyme remains active (figure 5-7 C). Sodium format has no effect on the stability of α-amylase (figure 5-7 B). A destabilizing effect on α-amylase can be observed if boric acid is added and the residual enzyme activity after 28 days is cut by half (figure 5-7 B). Stabilizing effects using boric acid are present with protease and especially lipase. In the latter case 50% of the enzyme are still active (figure 5-7 C). 4-FPBA and peptide aldehyde show quite similar effects on the stability of the enzymes tested.

Protease is significantly more stable in the presence of both additives, 4-FPBA and peptide aldehyde, and as a result a residual protease activity of 85% (peptide aldehyde) and 75% (4-FPBA) remains (figure 5-7 A). About 70% α-amylase are active after 28 d storage at 37 °C with the addition of peptide aldehyde or 4-FPBA, which is an increase compared to the pure enzyme (45%; figure 5-7 B). Both additives have no effect on the stability of lipase (figure 5-7 C). In addition to the storage tests, studies regarding the thermal stability of the three enzymes tested in presence of the small molecules have been performed (figure 5-8). Thermograms are shown in the appendix in chapter 11.2., figure 11-8.

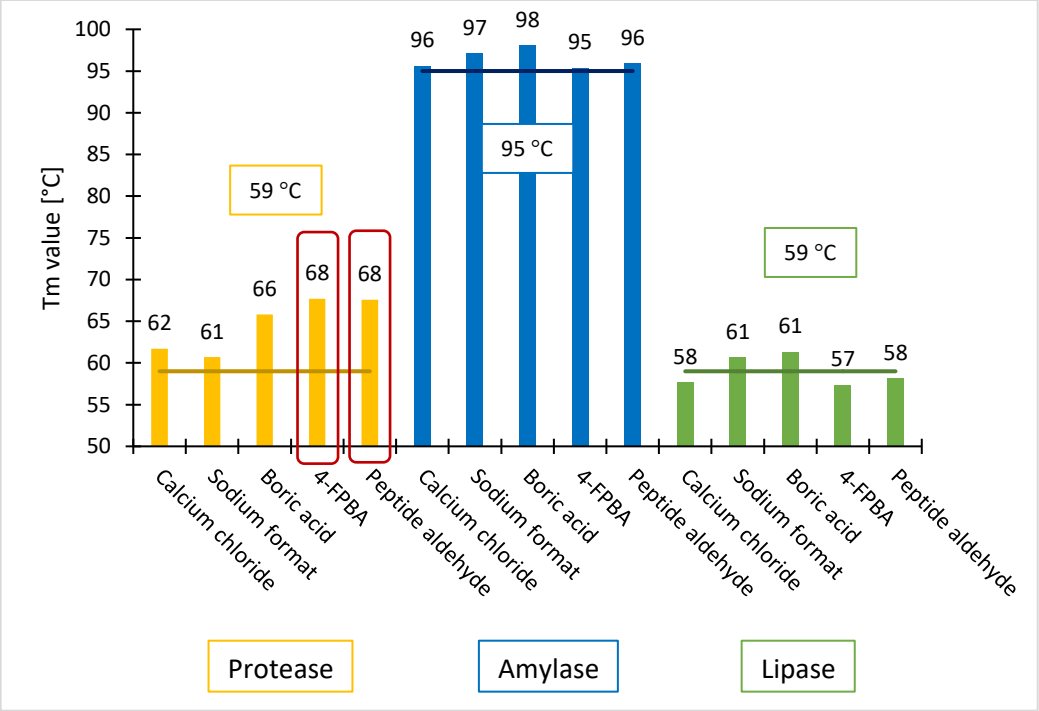


Figure 5-8: Overview of the results from nanoDSC experiments regarding thermal stability. Pure enzyme (line chart) and enzymes with small molecules additives (bar charts) are measured in a standard detergent formulation. Displayed are the T_m values obtained. Best results are marked by a red box. The average error for nanoDSC experiments in detergents is 1.5 K. For original data see appendix chapter 11.2.; figure 11-8.

Results regarding thermal stability show similar trends as observed in the storage tests. Protease stability can be greatly increased with the addition of 4-FPBA and peptide aldehyde (figure 5-8 red box). Whereas α-amylase and lipase are not significantly stabilized by addition of any of the established small molecules. Overall, the tests with the established small molecules for enzyme stabilization in liquid detergents have shown expected results. Protease is much more stable in presence of those additives, especially of the most used 4-FPBA and the new peptide aldehyde. The stability of the two other enzymes – α-amylase and lipase – is only little or not influenced resulting in a low enzyme activity after 28 d storage at 37 °C. Conducted experiments illustrate the need of stabilizing systems targeting

other enzymes than protease. Starting with small molecules addressing lipase, such strategies are tested in the present thesis.

5.2.2. Thermal Stability Measurements of Small Molecules

In first experiments, the four small molecules of choice – ACh, CACE, TEC and ATEC – are added to lipase in a concentration range from 0.5 to 4wt.-% and measured using nanoDSC. This screening is performed to observe enzyme stabilizing effects as well as concentration dependencies. 4wt-% is chosen as maximum concentration due to the formulation limits using standard detergent formulations. The results obtained are shown in figure 5-9. Thermograms are attached in the appendix in chapter 11.2., figure 11-9.

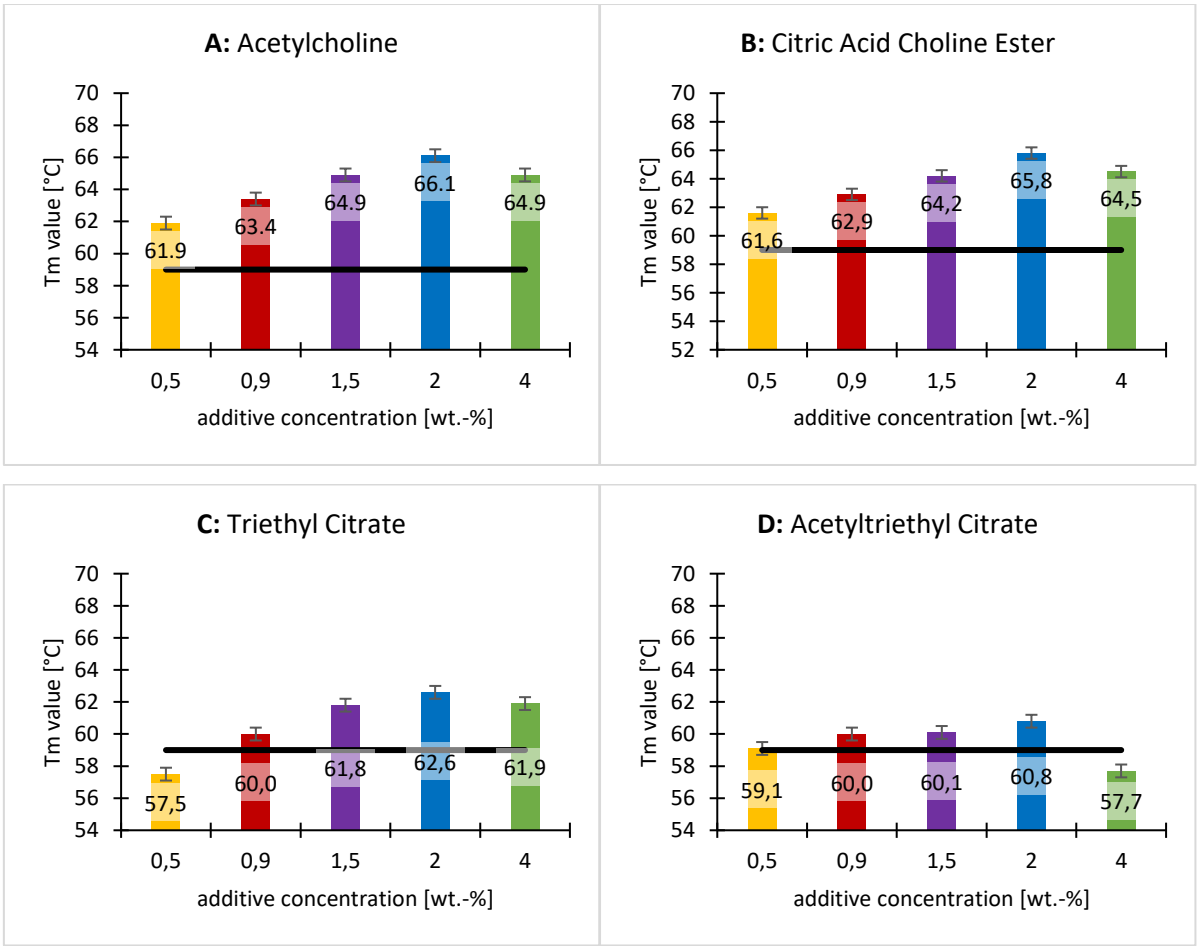


Figure 5-9: T_m values obtained from concentration screening of lipase thermal stability with the addition of acetylcholine (A), citric acid choline ester (B), triethyl citrate (C) and acetyl triethyl citrate (D). Measurements are conducted in a standard detergent formulation. Pure lipase shows a T_m value of 59 °C (black line). ● pure enzyme ● 0.5wt.-% additive ● 0.9wt.-% additive ● 1.5wt.-% additive ● 2.0wt.-% additive ● 4.0wt.-% additive. For original data see appendix chapter 11.2., figure 11-9.

Pure lipase has a T_m value of 59 °C dissolved in the standard detergent formulation used (figure 5-9). Addition of ACh leads to an increase of the thermal stability with a maximum of 66.1 °C using an ACh concentration of 2wt.-% (figure 5-9 A). The additions of CACE leads to a significantly enhanced thermal stability of lipase. With increasing additive concentration, the higher the resulting T_m value until a maximum with 2wt-% CACE and a T_m value of 65.8 °C is achieved (figure 5-9 B). A similar even though weaker trend is obtained with the addition of TEC. The presence of 2wt-% TEC results in a slightly increased T_m value of 62.6 °C (figure 5-9 C). Almost negligible is the stabilizing effect observed by the addition of ATEC. In the best case, the T_m value of lipase is shifted by about 2 K to 60.8 °C with a concentration of 2wt-% ATEC (figure 5-9 D). All four additives show the highest thermal stabilization effect with a concentration of 2wt-% and by comparison a reduced effect using a higher concentration of 4wt-%. One reason might be that at concentrations of 2wt-% a saturation of the lipase active site is reached. Higher concentrations lead to interactions between residual small molecules and other lipase parts resulting in a decreased conformational stability. Nevertheless, building on these promising results, storage tests in a standard detergent formulation using an additive concentration of 2wt-% have been performed. In the next chapter the result of storage tests can be found.

5.2.3. Testing of Small Molecules

The four additives tested – CACE, ACh, TEC and ATEC – show at least a small stabilizing effect regarding the lipase thermal stability in the concentration screening experiments (chapter 5.2.2.). In the following storage tests the small molecules are added with a concentration of 2wt-% to lipase in a standard detergent formulation. In addition, lipase stability in presence of protease is tested to evaluate if proteolysis can be prevented by these additives. Besides, protease and α-amylase are stored in a standard detergent formulation in presence of the small molecules. These experiments serve to investigate if the stability of the other enzymes – protease and α-amylase – is influenced positively or negatively. Washing performance tests against stained textiles are waived since the accessibility of lipase to substrates is not reduced by the presence of small molecules. Such experiments are only important for chemically modified, encapsulated or immobilized enzymes.

Results of storage tests regarding the stability and activity of lipase in presence of ACh, CACE, TEC and ATEC in a standard detergent formulation and in water with protease are shown in figure 5-10. Solutions for experiments in SDF are stored at 37 °C, tests against protease are stored at 22 °C.

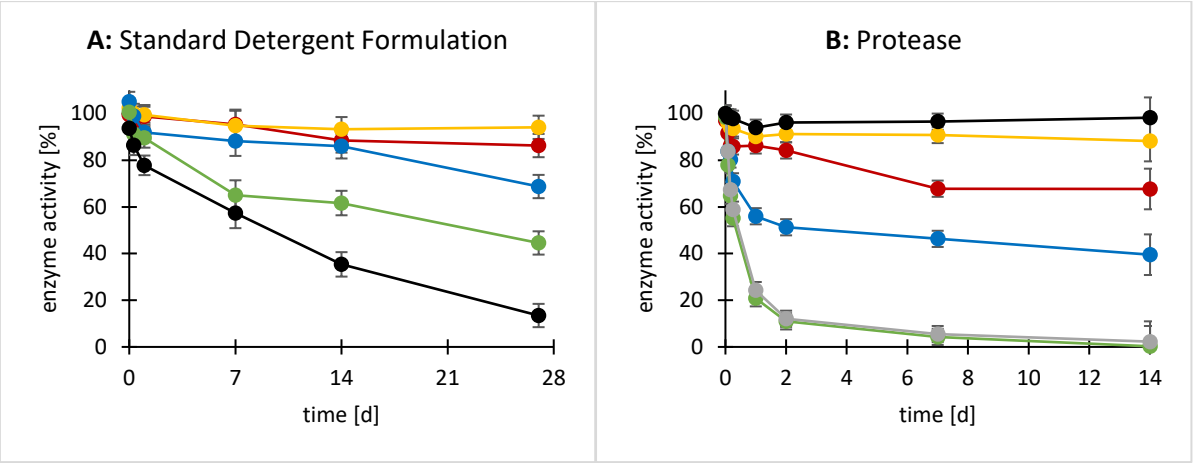


Figure 5-10: Graphical representation of the storage tests of lipase with the addition of 2wt.-% ACh, CACE, TEC and ATEC in a standard detergent formulation and in water against protease at room temperature. A: lipase in SDF. ● pure lipase ● CACE ● ACh ● TEC ● ATEC. B: lipase in water against protease. ● pure lipase ● CACE+protease ● ACh+protease ● TEC+protease ● ATEC+protease ● lipase+protease. Connecting lines are only guide to the eye.

After one month storage in an SDF at 37 °C, lipase shows a residual enzyme activity of about 15% (figure 5-10 A). The individual addition of the four small molecules results in an increase of the lipase stability. Especially the presence of 2wt-% CACE implies that the enzyme activity is almost completely retained after one month (94%). ACh gives similar results with a residual lipase activity of 86%. Minor stabilizing effects can be observed using TEC (69%) and ATEC (45%) (figure 5-10 A).

As discussed in chapter 5.2.3, lipase serves as a substrate for protease and for this reason the lipase stability is significantly reduced in the presence of protease – after two weeks at 22 °C almost no enzyme activity is observed anymore (figure 5-10 B, grey). The addition of 2wt.-% ATEC has no stabilizing effect on lipase (figure 5-10 B, green and grey) and the curves overlay exactly. However, 2wt.-% TEC, ACh and CACE can increase the stability of lipase in presence of protease significantly – the latter additive has the strongest effect, and, in this case, lipase shows almost an identical stability with and without protease (figure 5-10 B). By comparing the results in an SDF and against proteolysis, same trends can be observed. The stability of lipase is best preserved with the addition of CACE, followed by ACh. Rather moderate effects are caused by TEC and especially by ATEC. It should be mentioned that the protease stability in the experiments shown in figure 5-10 B is examined as well. Results are shown in figure 5-11.

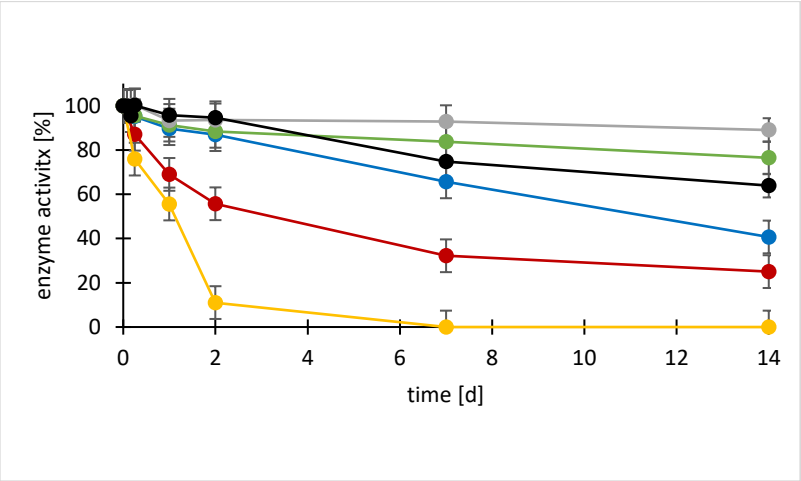


Figure 5-11: Graphical representation of the storage tests of protease with the addition of 2wt.-% ACh, CACE, TEC and ATEC in water with lipase at room temperature. The average error for pH measurements is 0.1 pH unit. ● pure protease ● CACE+lipase ● ACh+lipase ● TEC+lipase ● ATEC+lipase ● lipase+protease. Connecting lines are only guide to the eye.

Figure 5-11 shows that the protease stability is influenced by the presence of the four additives. Pure lipase (without the presence of stabilizers tested) ensures as a substrate that the protease activity is preserved. A quite similar situation appears with 2wt.-% ATEC. Conversely, TEC, ACh and especially CACE reduce the protease stability significantly. In the latter case, no protease activity is measured after two weeks (figure 5-11). Figure 5-11 and figure 5-10 show contrary results regarding the stability of protease and lipase. CACE, for example, increases the stability of lipase and decreases the stability of protease. In light of these results, it can be assumed that the increased lipase stability observed in figure 5-10 can be attributed to the defunctionalization of the protease. Protease is denatured by the presence of CACE, ACh or TEC before it can degrade lipase.

The influence of the four small molecules on the activity and thermal stability of protease and α-amylase is investigated further in an SDF. Results of the storage tests (37 °C) and of nanoDSC experiments are shown in figure 5-12. Thermograms are attached in the appendix in chapter 11.2., figure 11-10.

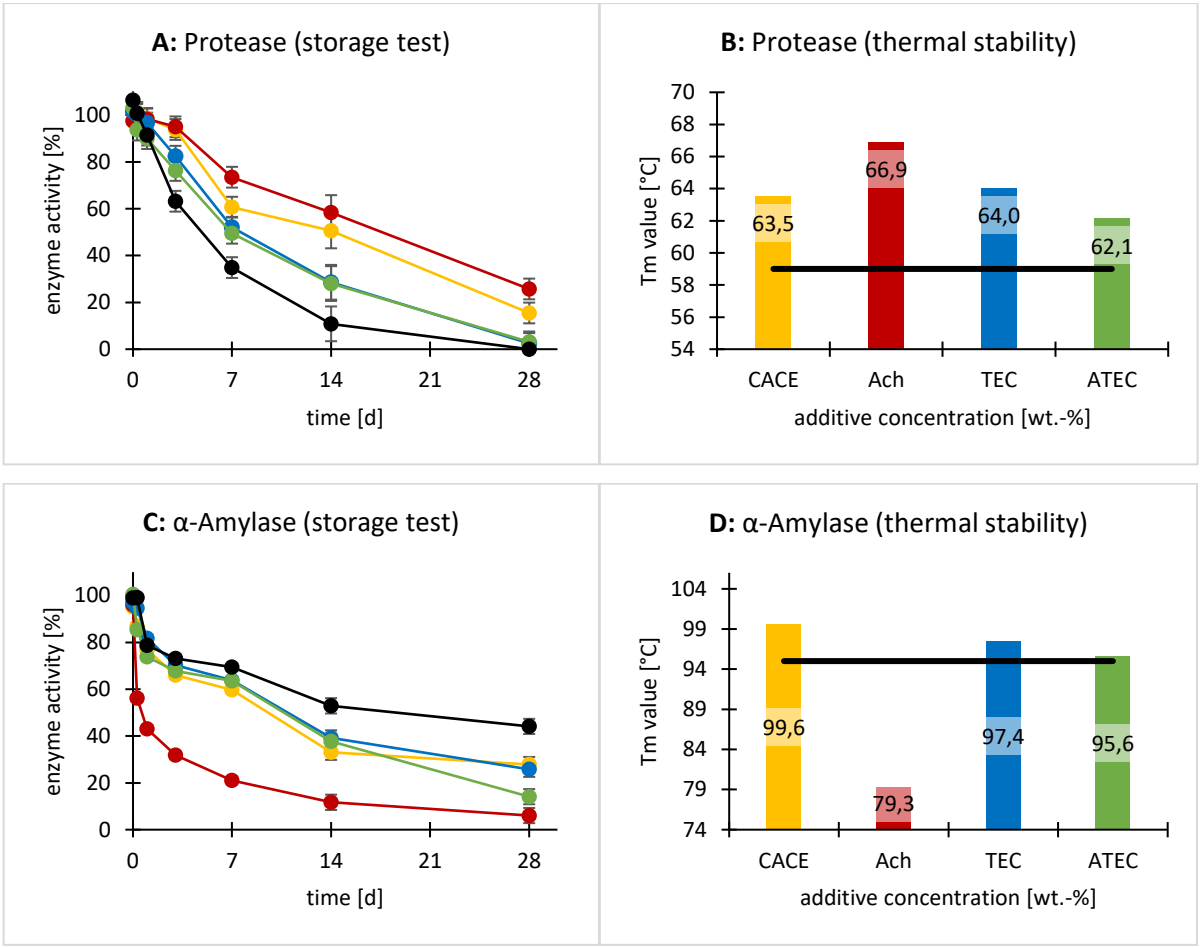


Figure 5-12: Results of the storage tests and the thermal stability experiments with the addition of ACh, CACE, TEC and ATEC in a standard detergent formulation. Storage tests is performed at 37 °C. Pure protease shows a T_m value of 59 °C and α-amylase of 95 °C (black lines). A: protease storage test, B: protease thermal stability, C: α-amylase storage test, D: α-amylase thermal stability. Connecting lines are only guide to the eye. ● pure enzyme ● CACE ● ACh ● TEC ● ATEC. The average error for nanoDSC experiments in detergents is 1.5 K. For original data see appendix chapter 11.2., figure 11-10.

Analyzing storage tests and thermal stability results, protease stability is increased due to the addition of the small molecules (figure 5-12 A&B). ACh leads to the highest residual enzyme activity (26%) after 28 d storage at 37 °C and a maximum T_m value shift of +8 K. Results obtained with CACE are slightly less stabilizing with 16% residual activity and a shift of about +5 K. The stabilizing effect on protease is further reduced with the addition of TEC and ATEC. Both small molecules lead to a residual enzyme activity of less than 5% after 28 days at 37 °C and smaller shift of the T_m value compared to ACh or CACE.

Based on the enzyme activities measured in the storage test of α-amylase, the stability of the enzyme is reduced in the presence of the four small molecules tested (figure 5-12 C). CACE, TEC and ATEC show similar residual enzyme activities resulting in about 20 to 25% after 28 days storage. This destabilizing effect is relatively low compared to pure α-amylase with 44% and to ACh, which ends up with an enzyme activity of 6%. The detrimental effect of ACh on α-amylase is observed in experiments

regarding thermal stability as well (figure 5-12 D). Here, the T_m value of the enzyme is reduced by about 15 K, if ACh is added, compared to the pure α-amylase. Contrary, CACE, TEC and ATEC increase the thermal stability of α-amylase slightly with a maximum shift of +5 K using CACE.

In total, the stability of lipase in storage tests against standard detergent formulations, as well as in nanoDSC experiments regarding thermal stability, can be increased by the addition of ACh, CACE, TEC and ATEC. ACh and especially CACE perform best so that the enzyme activity can be preserved at about 90% after 28 d storage at 37 °C in an SDF (figure 5-10). The results suggest that for best lipase stabilization in an SDF the charged quaternary amino group is essential. Stabilizing effects without a positively charged molecule (TEC and ATEC) are reduced. The difference between one and two quaternary amine groups (CACE and AC) is low with slightly higher enzyme activities in the presence of two positive charges.

The resistance of lipase towards proteolysis can be increased by the addition of TEC, ACh and especially CACE (figure 5-10). These trends are similar to the storage test in an SDF. ATEC as an additive has no stabilizing effect on lipase in this experiment. However, the enhanced stability of lipase is not a result of lipase protection, but of denaturing protease.

In a standard detergent formulation, stabilizing effects using the four small molecules with protease are obtained. The effects are not significant and unsatisfying compared to the established protease stabilizers on the market (4-FPBA or peptide stabilizer, see chapter 5.2.1.). Using ACh, protease shows a residual enzyme activity of 26% (figure 5-12 A), at the same time 85% are obtained by the addition of peptide aldehyde (figure 5-7 A). The third enzyme, α-amylase is not or negatively influenced by the addition of the four small molecules. Residual enzymes activities are lower compared to the pure enzyme whereas thermal stabilities are slightly increased with CACE, TEC and ATEC. A huge detrimental effect can be observed if ACh is added to α-amylase (figure 5-12 C&D). ACh leads to best stabilizing effects of the four small molecules for protease and to the by far worst stability of α-amylase. In that case, the family relationships are present. Protease belongs as lipase or AChE to the family of hydrolases with a catalytic triad, α-amylase has a different mode of action (chapter 2.2.). Therefore, it has been expected that positive stabilization effects decrease from lipase to protease and further to α-amylase.

In addition, simulations with ACh, CACE and TEC regarding the conformational stability of lipase and protease are conducted. Due to the complexity of a standard detergent formulation, the simulations are performed in water. However, the lid of lipase is closed in water and the active site is not accessible for the small molecules. On this account, the interactions between lipase and the small molecules are more restricted to the enzyme surface. Results regarding conformational stability of lipase and protease are shown in figure 5-13 and figure 5-14 respectively.

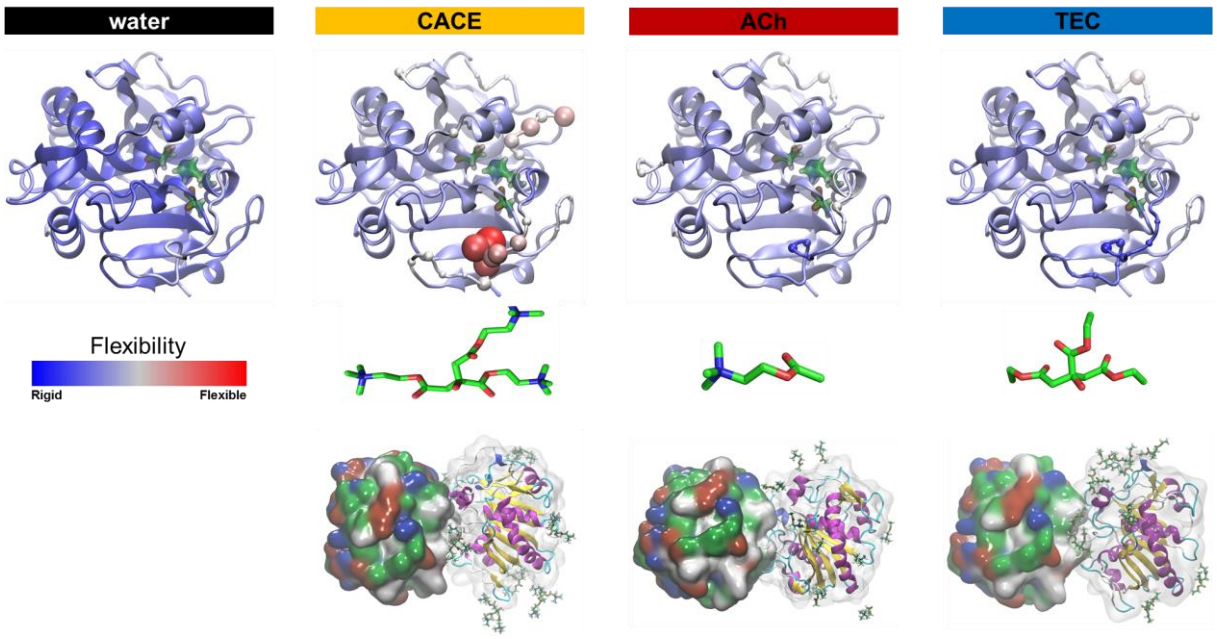


Figure 5-13: Simulation with CACE, ACh and TEC regarding the thermal stability of lipase. In the first row, the parts of lipase that are influenced by the additives are divided into more rigid (blue) and more flexible (red). In the second and last row the adsorption locations of the small molecules on the lipase surface are shown. Images by Priya Anand (BASF).

As shown in figure 5-13, TEC molecules tend to adsorb at loops that are located at the interface of the lipase dimer. The addition to the hydrophobic regions results in a prevented aggregation and dimerization of the enzyme. Furthermore, the rigidity and relating thereto the conformational stability of lipase is enhanced. Likewise, ACh additives (figure 5-13) increase the rigidity and conformational stability of lipase. Though, ACh molecules bind allocated to the enzyme surface and are not limited to the hydrophobic parts. CACE interacts with lipase allocated, but the enzyme flexibility is enhanced. Furthermore, the flexibility of protease, especially close to the active site, is increased by the addition of CACE (figure 5-14). Accordingly, the conformational stability of protease is significantly decreased by CACE. On the contrary, the presence of TEC increases the rigidity of protease due to allocated interactions with the enzyme (figure 5-14).

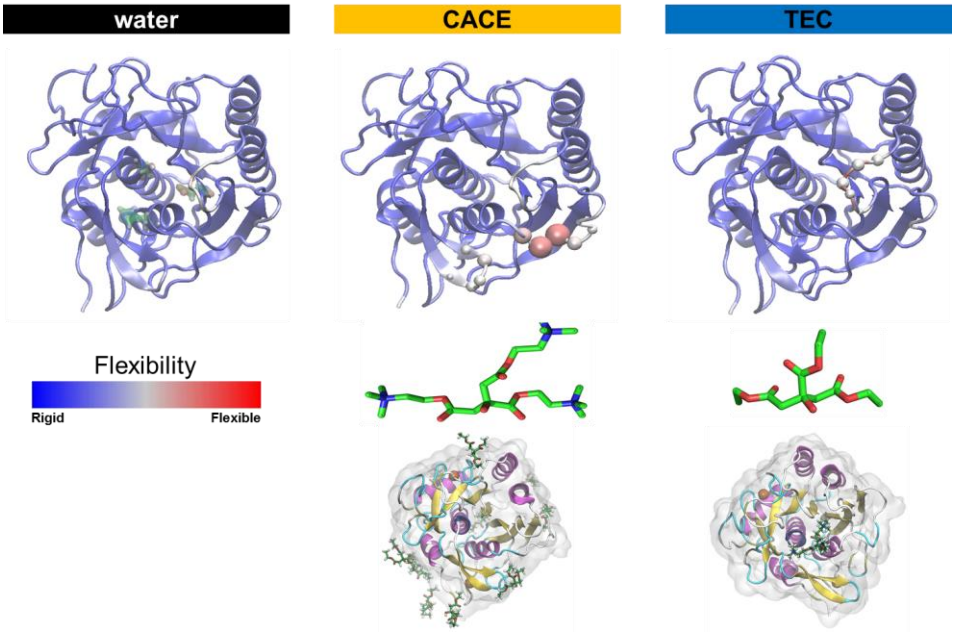


Figure 5-14: Simulation with CACE, ACh and TEC regarding the thermal stability of protease. In the first row the parts of protease that are influenced by the additives are divided into more rigid (blue) and more flexible (red). In the second and last row the adsorption locations of the small molecules are shown. Images by Priya Anand (BASF).

During the experiments in the SDF, the pH value of the lipase solutions stored is measured additionally to the enzyme activity. A drop in pH value is a hint of the ester bond cleavage present in all four small molecules and the subsequent release of an acid (citric acid or acetic acid). Measured pH value are plotted against the time in figure 5-15.

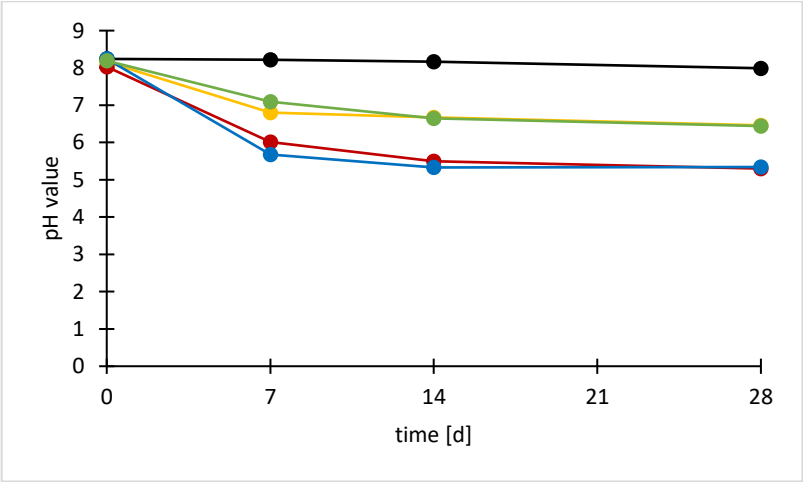


Figure 5-15: Measured pH values of storage solutions containing lipase and one of the small molecules tested respectively in a standard detergent formulation. Connecting lines are only guide to the eye. ● pure enzyme ● CACE ● ACh ● TEC ● ATEC.

The presence of all four small molecules results in a pH drop of the standard detergent formulation compared to pure lipase where the pH remains at 8 (figure 5-15). CACE and ATEC as well as ACh and

TEC behave quite similar resulting in final pH values of about 6.5 and 5.5 respectively. Ester bonding cleavage of ACh releases acetic acid, CACE and TEC citric acid and ATEC a mixture of acetic and citric acid. The original assumption that ACh interacts with the lipase active site without decomposition as a substrate must be rescinded. Interactions between lipase and the ester compounds result in cleavage of the ester bonds. A drop in pH value leads to a less effective washing performance of the surfactants and is therefore undesirable. A further question arises: are the stabilization effects observed a result of the pH drop? Previously considered nanoDSC measurements have been conducted without storage as direct measurement. Consequently, it can be assumed that the pH drop has not completely occurred so far and the stabilization effects in those experiments result from the presence of the intact additives. Nevertheless, to answer this question further, nanoDSC experiments with lipase in standard detergent formulations with adjusted pH values without additives are conducted. After a storage of 28 d at 37 °C thermal stabilities in presence of the additives are measured. In the latter experiments, a pH drop can be observed. Thermal stabilities obtained with an SDF at pH 5.5 and 6.5 as well as with additives after storage are shown in figure 5-16 A. In addition, hydrolysis of the esters has been simulated and lipase thermal stability is measured in presence of the concentrations of the decomposed ester alone and in a mixture. For instance, 2wt-% ACh are hydrolyzed to about 1wt.-% choline and 0.4wt.-% acetic acid. The thermal stability of lipase is measured in presence of 1wt.-% choline and 0.4wt.-% acetic acid respectively and a mixture containing both compounds (figure 5-16 B). Thermograms are attached in the appendix in chapter 11.2., figure 11-11.

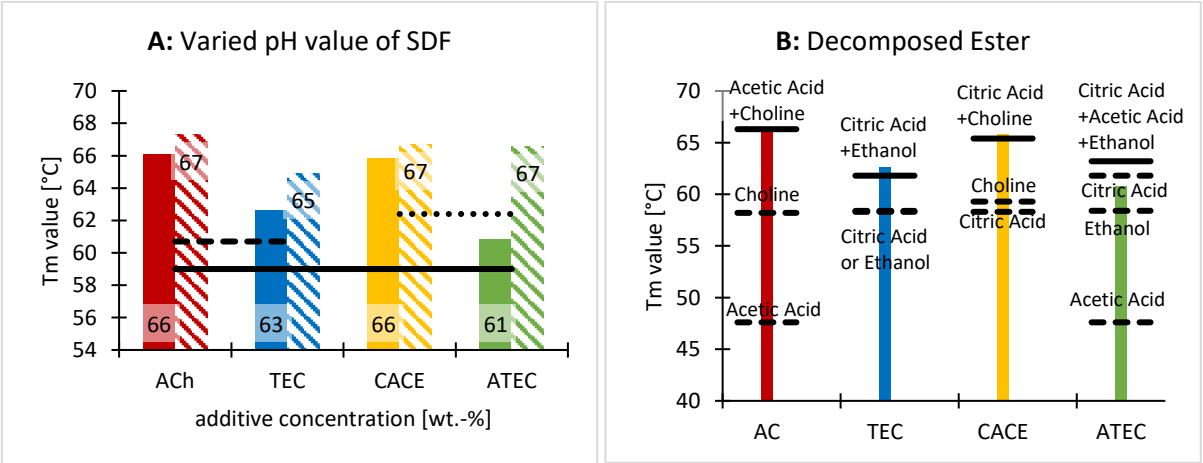


Figure 5-16: Further experiments regarding thermal stability with lipase in a standard detergent formulation. A: SDF normal (_____) with a adjusted pH of 5.5 (_ _ _ _) and of 6.5 (.....). Compared with the results obtained by the addition of 2wt.-% ACh, TEC, CACE and ATEC. Filled bars are direct nanoDSC measurements, striped bars are after 28 d storage at 37 °C. B: SDF with the addition of the hydrolyzed compounds. The compounds are measured separately (_ _ _ _) and mixed (_____). Acetic acid and choline for ACh, citric acid and ethanol for TEC, choline and citric acid for CACE and citric acid, acetic acid and ethanol for ATEC. The results are compared with previous results obtained by the addition of 2wt-% ACh, TEC, CACE and ATEC. The average error for nanoDSC experiments in detergents is 1.5 K. For original data see appendix chapter 11.2., figure 11-11.

Adjusting the pH value of SDF, it can be observed that the T_m value of lipase is increased at lower pH values (figure 5-16 A). At pH 8 to 8.5 a T_m value of 59 °C is measured, whereas at pH 6.5 half of lipase are denatured at 62.4 °C and at pH 5.5 at 60.7 °C. The pH adjusted SDFs show a lower thermal stability compared to the stored solutions with additives. After four weeks storage at 37 °C, samples containing ACh and TEC have a pH of 5.5 and the T_m measured has values of 67.3 and 64.9 °C respectively. In case of CACE and ATEC the pH value after storage period is at 6.5 and T_m values possess temperatures of 66.7 °C (CACE) and 66.6 °C (ATEC). Comparing the measured T_m values before and after storage, it is apparent that the values after storage are higher than before. This might be due to the drop in pH during the storage. However, in both cases – direct measurement and after storage – the thermal stabilities obtained are higher in presence of the additives than in the SDF at same pH value (figure 5-16 A).

Direct measurement of the ester compounds separately leads to reduced thermal stabilities in all four cases (figure 5-16 B). If the ester compounds are mixed the resulting T_m values are in accordance with the values obtained by measuring the intact ester compounds. 2wt.-% ACh shows in a direct measurement a T_m value of 66.1 °C. The mixture of 1wt.-% choline and 0.4wt.-% acetic acid results in 66.3 °C. Similar results are obtained for TEC (62.6 °C ester, 61.8 °C mixture), CACE (65.8 °C ester, 65.4 °C mixture) and ATEC (60.8 °C ester, 63.2 °C mixture). This could be explained by the fact that lipase is a catalyst that can accelerate the equilibrium of acid and alcohol to ester. Lipase can form a complex with the compounds and stabilizing effects compared to the ester are obtained. Contrary to the hydrolysis of the esters in the measurements with ACh, CACE, TEC and ATEC is that the pH drop has not accorded at the direct measurements in the nanoDSC and the thereby observed enhanced enzyme stabilities. It can be assumed that an equilibrium between hydrolyzed ester and intact ester occurs after about two weeks storage resulting in a constant pH value (figure 5-15).

Overall, figure 5-16 shows that the stabilizing effects observed can not only be attributed to the drop in pH value. In the running BASF project, it was possible to prevent the decrease in pH while maintaining the stabilization effect by the addition of appropriate amine compounds.

Chapter 5.2. can be summarized that various new and established small molecules have been tested regarding their properties to increase the stability of enzymes, especially lipase, in a standard liquid detergent formulation. As mentioned before, established stabilizers target protease and the inhibition of proteolysis. Lipase and α-amylase are only insufficiently stabilized by those additives. Therefore, four completely new small molecules, acetylcholine, citric acid choline, triethyl citrate and acetyl triethyl citrate are tested as stabilizers for lipase. With a concentration of 2wt.-% all four ester compounds have been able to increase the stability of lipase in storage tests as well as in

measurements regarding the thermal stability. Best results have been obtained with acetylcholine and citric acid choline ester. In the case of the two compounds bearing at least one quaternary amine, after 28 d storage at 37 °C in standard detergent formulation a residual enzyme activity of about 90% can be measured (pure lipase with 15%). Due to control experiments conducted, the stabilizing effects by the four small molecules are not only the result of a pH drop observed, but by the ester compounds.

In experiments regarding the stability of lipase towards proteolysis, an increased lipase stability in the presence of the two choline esters due to a denaturing of protease has been observed. Interestingly, the denaturing effects on protease only occur in water and contrarily not in an SDF. Here, the protease stability is increased slightly by the presence of the four small molecules. Whereas α-amylase is not stabilized and even significantly destabilized by acetylcholine.

With the exception of CACE – lipase, the simulations explain the results regarding lipase and protease stability observed in nanoDSC experiments and storage tests. TEC and ACh increase the rigidity of lipase which has a positive effect on the conformational stability. Similarly, the stability of protease is enhanced by TEC. The destabilizing effect of CACE on protease results from an increased flexibility of the enzyme.

In table 5-2 all small molecules tested are listed and the influence on the three enzymes underlining lead candidates is shown.

Table 5-2: Summary of small molecules stabilizing protease, α-amylase and lipase in standard liquid detergent formulations. Destabilizing effect, no effect, stabilizing effect, lead candidate.

Small Molecule	Protease	α-Amylase	Lipase
Calcium chloride			
Sodium format			
Boric acid			
4-FPBA			
Peptide aldehyde			
Acetylcholine			
Citric acid choline ester			
Triethyl citrate			
Acetyl triethyl citrate			

Finally, it has been shown that in contrast to protease, other enzymes like lipase and α-amylase are insufficient stabilized by the established small molecules. With citric acid choline ester, a promising compound that increases the stability of lipase significantly without huge detrimental effects regarding the other enzymes has been identified.

The work shown in this chapter has been supported by Stephan Hüffer (idea provider, synthesis of CACE and ATEC) and Priya Anand (modelling). Enzyme measurements regarding thermal stability and enzyme activity are made by me. Parts of the work are in a patent application: Hüffer, S.; Garcia-Marcos, A.; Kübelbeck, S.; Baier, G.; Spangenberg, O.; Compounds stabilizing hydrolases in liquids. Date of the Invention: 14.02.2018. Invention Status: filed (10.10.2018), application no. EP18199698.4.

5.3. Enzyme-Polymer Conjugates

One strategy tested to enhance enzyme stability in a liquid detergent is the synthesis of enzyme-polymer conjugates. Figure 5-17 shows the general approach beginning with the covalent conjugation and reaction control via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC). The synthesis is followed by the purification of the reaction mixture. Depending on the reaction yield, purification is performed by dialysis (no pure enzyme left in reaction control) or by preparative field-flow fractionation (FFF; pure and not converted enzyme is left). After purification enzyme-polymer conjugates are freeze-dried before the total amount of protein is determined by bicinchoninic acid assay (BCA assay) and by measuring absorption at 280 nm (UV assay). In the next steps, residual enzyme activity and thermal stability of the enzyme after conjugation are determined. In storage tests enzyme-polymer conjugates are examined with respect to a stabilization effect compared to the pure enzyme in a liquid standard detergent formulation. Finally, lead candidates are investigated whether the enzyme covalently linked to a polymer still performances towards stains on real textiles (figure 5-17).

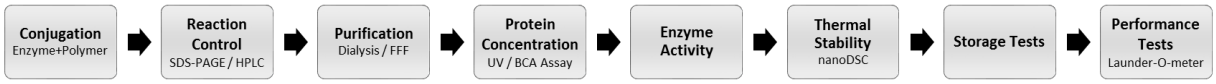


Figure 5-17: General approach for synthesis, isolation, characterization and testing of enzyme-polymer conjugates.

5.3.1. Synthesis of Enzyme-Polymer Conjugates

Prior to the actual synthesis, the number of reactive and accessible enzyme amino acid residues is determined theoretically by consideration of the location of lysine residues and experimentally by using the reagent trinitrobenzenesulfonic acid (TNBS, see chapter 4.2.2.). The total amount of lysine residues is derived from the amino acid sequence of the enzymes tested. By computer simulation it is possible to visualize amino groups located on the enzyme surface. Via TNBS assay the amount of accessible amino groups is determined by calibration with L-Alanin. In table 5-3 the total amount of lysine residues as well as the number of accessible amino groups by computer simulation and by TNBS assay are shown for protease, α-amylase and lipase.

Table 5-3: Listing of the total amount of lysine residues and the number of accessible amino groups determined by consideration of the location of lysine residues and TNBS assay for protease, α-amylase and lipase.

Enzyme	Total Amount of Lysine Residues	Accessible Amino Groups Computer Simulation	Accessible Amino Groups TNBS Assay
Protease	5	3	2.8
α-Amylase	28	8	7.8
Lipase	7	7	7.3

The results obtained by TNBS assay are in accordance with the results by computer simulation. While amylase and lipase bear a similar amount of accessible amino groups on their surface, protease possess only a small number.

In order to synthesize enzyme-polymer conjugates several different polymers carrying various functional groups reactive toward covalent coupling with amino groups have been tested (see chapter 2.5.). These include two monofunctional PEGs (mPEG-aldehyde and mPEG-NHS, both 5 kDa) and two multifunctional polysaccharides (maltodextrin- and CMC-aldehyde). It is worth noting that aldehyde (polymer) and amine (enzyme) are coupled via reductive amination to a Schiff base, which is further reduced irreversible by sodium cyanoborohydride to a secondary amine (see figure 2-12). The reaction conditions are designed such that the conjugation is random and not selective. In general, the reaction is controlled, and the conversion rates are determined using SDS-PAGE and HPLC. Corresponding gel images and elugrams are shown in the appendix (chapter 11.3., figure 11-12 to 11-15). For α-amylase and lipase, conjugation occurred very efficiently with conversion rates of 100% for all polymers shown (table 5-4). This means that at least every enzyme molecule is conjugated to at least one polymer chain. In contrast to the efficient conjugation of α-amylase and lipase, using protease a complete conversion is only observed for the two polysaccharides maltodextrin- and CMC-aldehyde. Whereas the activated PEGs showed a conversion of 45% (table 5-4). The difference between protease and the two other enzymes, may be justified by the lower number of accessible amino groups on the protease surface (three lysine) compared to the surface of α-amylase and lipase (eight respective seven lysine) (table 5-3).

Table 5-4: Conversion rates of protease, α-amylase and lipase with mPEG-ald, mPEG-NHS, maltodextrin-ald and CMC-ald. Conversion relates to the percentage of modified enzyme where every enzyme molecule is at least covalently linked to one polymer molecule. Values are determined by HPLC (for original data see appendix chapter 11.3., figure 11-12 to 11-15).

Enzyme	mPEG-aldehyde	mPEG-NHS	Maltodextrin-aldehyde	CMC-aldehyde
Protease	45%	44%	100%	100%
α-Amylase	100%	100%	100%	100%
Lipase	100%	100%	100%	100%

It is striking that gel images obtained by the reaction of enzyme with PEG derivatives show a clear demarcation between native and conjugated enzyme, while gel bands with polysaccharides are little or not at all shifted to higher molar masses (see appendix chapter 11.3., figure 11-12 to 11 -14). A possible reason for this can be the potential multipoint attachment of polysaccharides to the enzymes. Differences between single and multipoint attachment are likewise visible via asymmetric flow field flow fractionation (AF4) measured hydrodynamic diameter. Pure α-amylase has a size of about 3 nm. Conjugated to maltodextrin-aldehyde the diameter remains unchanged, whereas conjugation with mPEG-aldehyde results in an increase to 6 nm. As maltodextrin possess the higher molar mass (20 kDa) compared to mPEG-aldehyde (5 kDa), it is likely that differences observed can be attributed to the distinct types of attachment.

To remove remaining unmodified enzyme, non-attached polymer and reducing agent where used, the reaction solutions are purified by dialysis or by FFF. Purified and diluted enzyme-polymer conjugates are freeze-dried, before further characterization.

5.3.2. Characterization of Enzyme-Polymer Conjugates

Initially, the protein concentration of the purified and lyophilized enzyme-polymer conjugates is determined using two different assays as mentioned in previous chapters. Thereby, besides active enzyme, denatured and inactive enzyme is captured as well using those assays. The amount of active enzyme is measured using enzyme specific activity assays. Using the total protein concentration and the amount of active enzyme, it is possible to calculate the percentage of enzyme that retains itsactivity despite the conjugation. Furthermore, the total yield being the product of conversion and enzyme activity, can be calculated. Results obtained are displayed in figure 5-18.

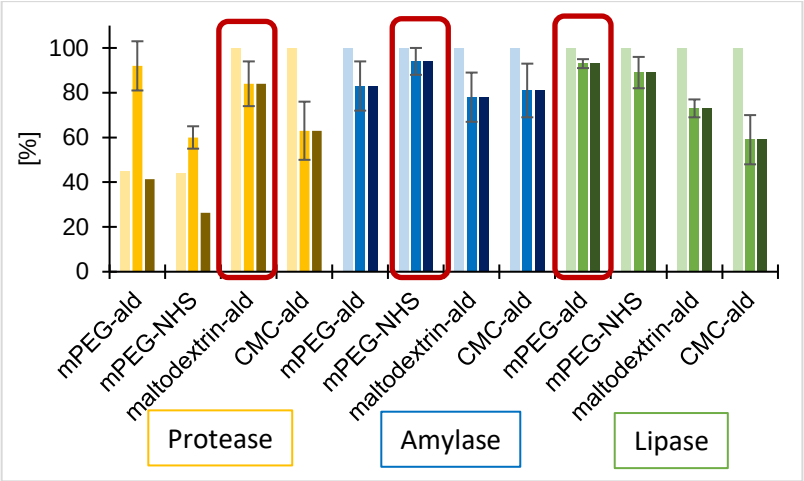


Figure 5-18: Diagram with the conversion rate, enzyme activity and total yield of enzyme-polymer conjugation reactions between protease, α-amylase and lipase and four different polymers. Best results are marked by a red box. ■ conversion rate ■ enzyme activity ■ reaction yield.

As clearly visible in figure 5-18, protease retained a maximum enzyme activity and a maximum yield upon conjugation with mPEG-aldehyde (92%; yield: 41%) and maltodextrin-aldehyde (84%; yield: 84%). Conjugation reactions with α-amylase result in a yield of at least 80% to 94% (mPEG-NHS). Lipase keeps about 90% of its initial activity when conjugated to the PEG derivatives. The experiments show that the best result regarding yield is obtained for protease and maltodextrin-ald (84%), α-amylase and mPEG-NHS (94%) as well as lipase and mPEG-ald (89%). In general, best result are obtained for α-amylase, showing a minimum yield of at least 78%.

Investigation of the number of polymers covalently linked to one enzyme molecule is implemented by AF4 and TNBS assay. Using AF4 the molar masses of enzyme-polymer conjugates are measured and with the knowledge of the molar mass of the pure polymers and enzymes the number of polymers attached to one enzyme can be calculated. Elugrams are shown in the appendix in chapter 11.3. (figure 11-16 to 11-18) The number of free and unconjugated amino groups is determined via TNBS assay, recalculation using the total amount of amino groups results in the desired number as well. Figure 5-19 shows the calculated number of polymers covalently linked by both methods for all three enzymes.

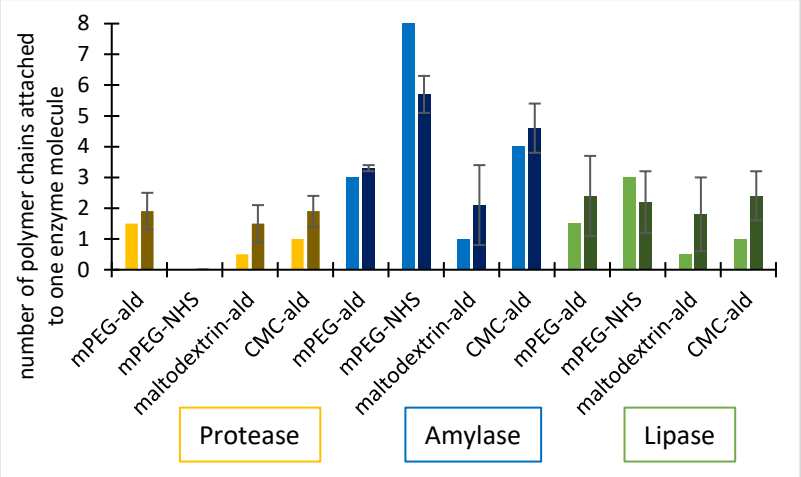


Figure 5-19: Graphic representation of the number of polymer chains attached to one enzyme molecule. ■ AF4 ■ TNBS assay.

The results regarding the number of polymer chains obtained by AF4 and TNBS assay are in most cases similar and follow the same trends. Conjugation of protease results to a maximum of one to two polymer chains per enzyme molecule. Whereas α-amylase and lipase carry up to eight or three polymer chains, respectively (figure 5-19). Consequently, at least half of the accessible amino groups of all three enzymes are conjugated with a polymer while the other half remains unmodified.

Using differential scanning microcalorimetry the thermal stability of the enzyme-polymer conjugates is determined. Therefore, native enzymes, enzymes covalently linked to polymers and unmodified enzymes in presence of polymers are exposed to the temperature program of the nanoDSC. It is investigated if the covalent attachment of a polymer increases the enzyme stability when heated up to 110 °C. In a nutshell, it is checked, if enzyme-polymer conjugates show a higher T_m value compared to the unmodified enzyme alone or rather in presence of the polymers without covalent attachment. Received T_m values are plotted in figure 5-20 and the corresponding thermograms can be found in the appendix in chapter 11.3., figure 11-19. In figure 5-20 the line charts outline the T_m value of the pure enzymes. Reference experiment with polymers without attachment are represented in lighter colors and enzyme-polymer conjugates with darker colors in form of bar charts.

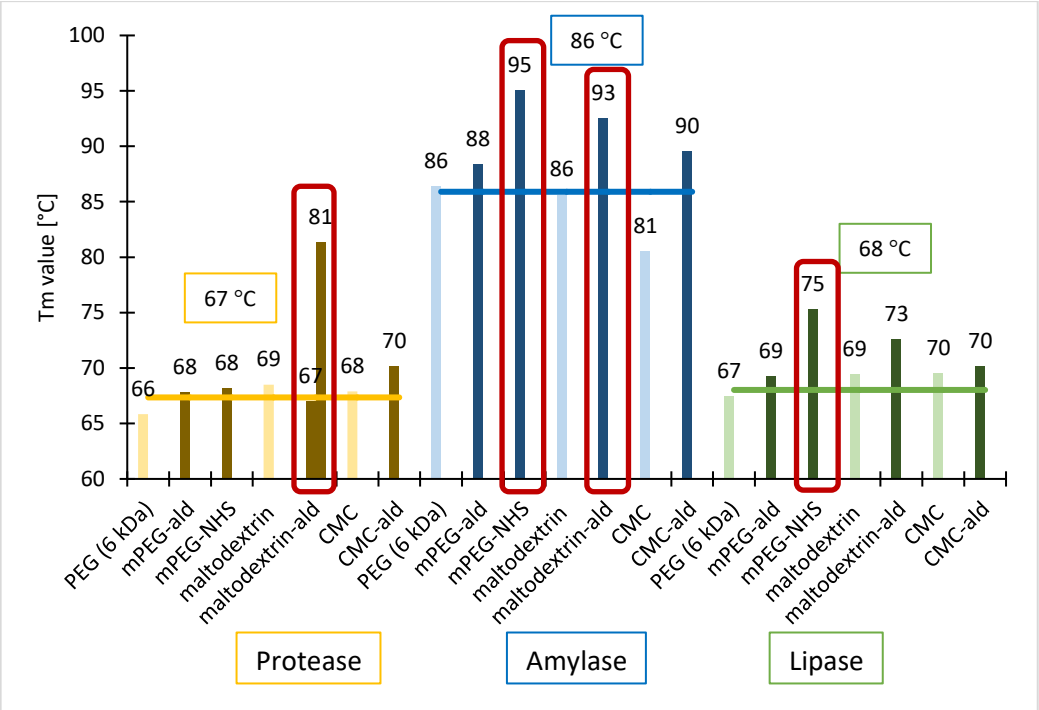


Figure 5-20: Overview of the results from nanoDSC experiments regarding thermal stability. Pure enzyme (line chart), enzyme-polymer conjugates (dark colors bar chart) and enzymes in presence of polymers without covalent attachment (light colors bar chart) are measured in phosphate buffer (100 mM, pH=8). Displayed are the T_m values obtained. Best results are marked by a red box. The average error for nanoDSC experiments in aqueous solutions is 0.5 K. For original data see appendix chapter 11.3., figure 11-19.

In phosphate buffer (100 mM, pH=8) protease shows a T_m value of 67 °C. A substantial stabilization occurs if protease is conjugated to maltodextrin-aldehyde. Here, the thermogram shows a bimodal shape with the main peak located at 81 °C. No detectable influence on the thermal stability of protease can be observed with all other conjugates as well as in presence of polymers without conjugation (figure 5-20). Under the present conditions α-amylase possess a T_m value of 86 °C. Conjugation with mPEG-NHS (95 °C) or maltodextrin-aldehyde (93 °C) results in a significantly increased thermal stability. Likewise, albeit weaker conjugates with mPEG-aldehyde (88 °C) and CMC-aldehyde (90 °C) enhance the thermal stability of α-amylase. Whereas the presence of polymers without conjugation has no positive impact on thermal stability; especially in case of CMC (82 °C; figure 5-20). Lipase shows a T_m value of 68 °C and an altered thermal stability after conjugation with mPEG-NHS (75 °C) or maltodextrin-aldehyde (73 °C). No detectable influence on lipases thermal stability is observed with all other conditions tested (figure 5-20).

In general, the presence of polymers without covalent attachment to the enzymes shows no positive effect on enzyme´s thermal stability. The reason for this may be the reduced polarity in case of PEG resulting in an interaction with hydrophobic amino acids, an initiation of enzyme unfolding and finally in a reduced T_m value.[307] Stabilizing effects regarding thermal stability caused by polymer conjugation can be attributed to an increased rigidity of a conjugated enzyme. A more rigid enzyme shows a

reduced structural dynamic which can protect against conformational changes and temperature induced unfolding.[180] Additionally, a polymer chain shows a shielding effect which protects an enzyme sterically against autolysis and prevents dissociation of an enzyme.[308] Absent effects despite conjugation can derive from a compensation of both described pronounced hydrophobic interaction and increased rigidity.[307] At least, it is considered that the thermal stability of an enzyme-polymer conjugate depends mainly on the number of polymer chains attached and is independent of the polymers molar mass. A saturation regarding thermal stability is reached after a certain amount of polymers is conjugated to the enzyme.[180] Another factor affecting enzymes´ thermal stability is the specific local surrounding of the polymer on the enzymes´ surface as well as the location of conjugation.[309] Here, the conjugation of α-amylase with mPEG-NHS results in a significantly increased thermal stability, whereas mPEG-aldehyde leads to no stabilizing effect.

Thermal stability of all enzymes tested is enhanced by conjugation with maltodextrin-aldehyde. Polymers with multiple attachment points to one enzyme molecule can conserve the native structure and prevent a denaturation. It is worth noting that for protease the peaks in nanoDSC of the conjugates are narrower than for the pure enzyme. The T_m value for protease-mPEG-aldehyde conjugate for instance is the same as for pure protease, but the onset temperature at which the denaturation of the enzyme starts is shifted to higher temperatures with 8 K. This strongly indicates a protection of protease against autoproteolysis.

5.3.3. Testing of Enzyme-Polymer Conjugates

In the first place, the synthesized and characterized enzyme-polymer conjugates are tested in a liquid standard detergent formulation. The storage tests are performed with the conjugates as well as in the presence of polymers without attachment at room temperature for defined time intervals. As shown in chapter 5.1.2. the conditions in a liquid detergent formulation are extremely harsh for enzymes. Results for reference samples containing non-covalently linked polymers are not shown. But in general, no enzyme stabilizing effect is observed under these conditions. In figure 5-21 A to C are the results of the storage tests with enzyme-polymer conjugates are displayed. It has to be mentioned that the experiments in chapter 5.1.2. have been performed with preformulated enzymes, resulting in higher enzyme stabilities. Here, the pure enzymes are purified by dialysis to ensure a better comparability with the dialyzed enzyme-polymer conjugates. For protease only the two conjugates showing a conversion of 100% are tested – maltodextrin-aldehyde and CMC-aldehyde.

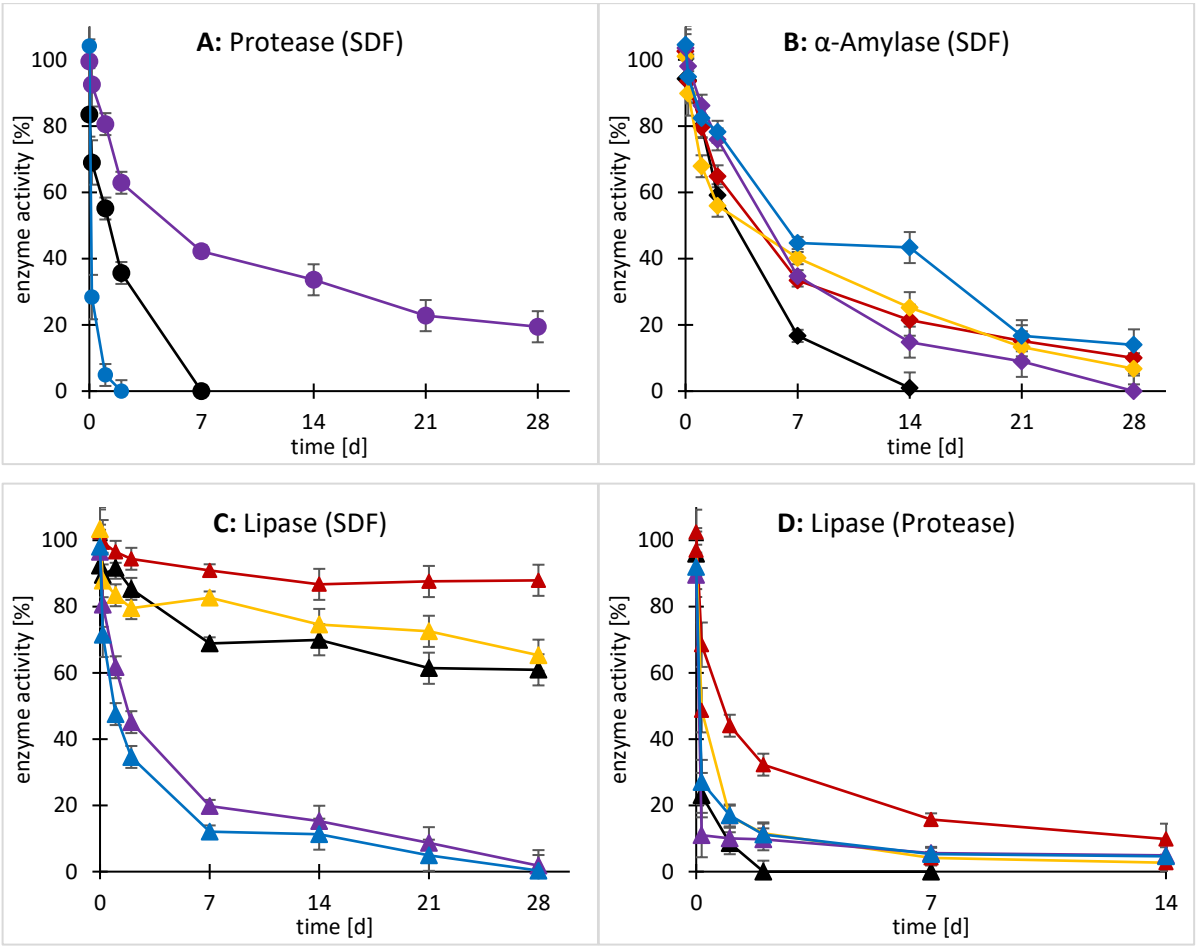


Figure 5-21: Graphical representation of the storage tests of enzyme-polymer conjugates in a standard detergent formulation and in water against protease at 22 °C. A: protease in SDF, B: α-amylase in SDF, C: lipase in SDF, D: lipase in water against protease. Connecting lines are only guide to the eye.
● pure enzyme ● conjugate with mPEG-aldehyde ● conjugate with mPEG-NHS ● conjugate with maltodextrin-aldehyde ● conjugate with CMC-aldehyde.

Pure protease is very sensitive to storage in a liquid standard detergent formulation (SDF) resulting in a complete activity loss after a storage period of seven days. Conjugated to CMC-aldehyde protease denatures even faster than as the native enzyme (two days). A noticeable stability increase is observed if protease is covalently linked to maltodextrin-aldehyde, with 20% of protease being still active after four weeks of storage in a detergent formulation (figure 5-21 A). Compared to protease, α-amylase is more stable in an SDF. Nevertheless, after a storage period of two weeks no residual protease and α-amylase activity is observed for the pure enzymes. All four α-amylase-polymer conjugates increase the α-amylase stability up to three weeks. The conjugate with CMC-aldehyde shows the most pronounced stabilization effect and after two weeks 40% of α-amylase activity is retained (figure 5-21 B). Interestingly, lipase is significantly more stable in the liquid detergent formulation and the pure enzyme possess after four weeks storage an activity of 60%. This stability is greatly reduced if lipase is conjugated to polysaccharides. The covalent attachment of maltodextrin- and CMC-aldehyde leads to a complete activity loss after four weeks storage. Nevertheless, conjugation

with mPEG-aldehyde and mPEG-NHS results in a slightly increased lipase stability and after four weeks storage about 90% activity are maintained (figure 5-21 C).

Despite the storage tests in liquid standard detergent formulations, lipase-polymer conjugates are tested in water against autoproteolysis by protease (figure 5-21 D). After two days storage pure lipase is degraded and completely inactive. Conjugation of lipase to mPEG-NHS leads to a significant enzyme stabilization and after two weeks at least 10% of the lipase are still active.

In analogy, to observed thermal stability effects, an enhanced stability of an enzyme-polymer conjugate in a liquid standard detergent formulation results from an increased rigidity of the enzymes due to the linkage to polymer chains. In addition, shielding caused by hydrogen bonds to the polymer around the enzymes gives a stabilizing effect.[180] Based on the conjugation to lysine residues, the number of positively charged functional groups on the enzyme surface which can interact with negatively charged anionic surfactants is reduced. The stabilizing effect of lipase conjugation regarding autoproteolysis can be attributed to the modification of the lipase surface resulting in a less suitable substrate for protease. Interestingly, the polymers have partially a completely different effect on the individual enzymes. CMC-aldehyde, for instance, stabilized α-amylase on the one side. But on the other side CMC-aldehyde strongly destabilize protease and lipase.

Due to the risk of a steric blockade of the enzymes active site by a covalently attached polymer, performance tests are necessary to prove that the active site is still accessible for real stain removal on textile fibers. On this account, two selected α-amylase conjugates are evaluated in a performance test. In these tests three different enzyme concentrations of pure α-amylase as well as α-amylase conjugated to mPEG- and maltodextrin-aldehyde are washed in a launder-o-meter at 40 °C together with dirty clothes for 30 minutes. The cloth is soiled with defined maize and rice starch stains, so that an analysis regarding their L* value is possible. Thereby a photometer measures the L* value before and after the washing process and the washing success – brightness of textile – can be quantified. In figure 5-22 the resulting dL* value is plotted against the enzyme concentration (ppm).

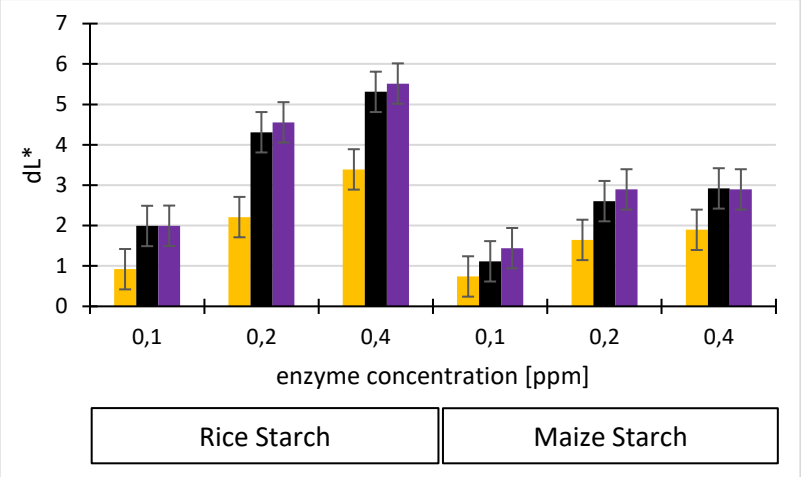


Figure 5-22: Washing performance of pure α-amylase, α-amylase-mPEG-aldehyde and α-amylase-maltodextrin-aldehyde conjugate. Washing tests are performed in a liquid standard detergent formulation at 40 °C with three different enzyme concentrations and two types of stain (rice and maize starch). ● pure enzyme ● conjugate with mPEG-aldehyde ● conjugate with maltodextrin-aldehyde.

It emerges that pure α-amylase and the enzyme conjugated with maltodextrin-aldehyde show comparable and good performance. In contrast, α-amylase covalently linked to mPEG-aldehyde shows a decreased performance of almost down to 50% as compared to the pure enzyme. One reason for this could be a shielded active site of the enzyme resulting in lowered the ability to remove stains on textiles.

Chapter 5.3. can be summarized as follows: the synthesis of enzyme-polymer conjugates using laundry-relevant enzymes is possible as described in literature. In particular the conjugation with α-amylase and lipase is easily achieved. Both enzymes possess more amino groups on their surface compared to protease for which a complete conversion with the PEG derivatives could not be reached. Despite the chemical modification, the conjugated enzymes retain their activity in large part and at least of 60%. It has been attempted to attach as many polymers as possible to one enzyme and at least a minimum of one to two polymer chains is conjugated per enzyme molecule. Conjugates with maltodextrin-aldehyde could especially improve thermal stability of the enzymes. Regarding the application in a liquid detergent, all three enzymes could be stabilized in a standard detergent formulation over short term of four weeks using conjugation to a polymer. And finally, in a washing performance test α-amylase conjugated with maltodextrin-aldehyde shows the same cleaning power as the unmodified enzyme. In table 5.5 the lead candidates for each enzyme at important characterization steps are shown.

Table 5-5: Tabular overview on the lead candidates of each enzyme at several steps of the approach. ● conjugate with mPEG-aldehyde ● conjugate with mPEG NHS ● conjugate with maltodextrin aldehyde ● conjugate with CMC aldehyde.

Enzyme	Conversion rate	Enzyme activity	Number of polymer chains attached	Thermal stability	Storage tests
Protease			-		
α-Amylase					
Lipase					

The synthesis of protease-polymer conjugates using the amino groups of the enzyme is problematic, so that only a small number of polymer chains can be attached to the enzymes surface. Using polysaccharides with more than one reactive group instead of the monofunctional PEGs the conjugation is feasible and stabilizing effects are observed. For α-amylase PEGylation and glycosylation enhance the enzyme stability regarding temperature and surfactants. However, in performance tests only the polysaccharide conjugate retains the enzymes performance. Interestingly, lipase conjugates with PEG score better than conjugates with polysaccharides. Lipase is – compared to the two other enzymes – more hydrophobic and therefore prefers the more hydrophobic polymer PEG.
In sum, it has been shown that the synthesis of enzyme-polymer conjugates represents an effective method for stabilizing enzymes against temperature and detergents up to four weeks. The stabilizing effect in a complete liquid standard detergent formulation is a newfound learning. Based on this results enzyme conjugation is of great interest for the detergent industry.

Many colleges have been contributed to the topic enzyme-polymer conjugates. Computer simulations regarding the number of amino groups on the enzymes surface has been done by Eduard Schreiner and Priya Anand. Harald Keller was idea provider for the oxidation of the polysaccharides and Jules Mikhael as well as Sven Machauer performed fractionating experiments. Janina Berndt is responsible for the washing performance tests. Finally, Walter Weishaar assisted with SDS-PAGE. The remaining work (synthesis, HPLC, dialysis, nanoDSC, protein concentration, enzyme activity, storage tests) has been part of my work. Parts of the work are published in Kübelbeck, S.; Mikhael, J. *et al.*, Enzyme-Polymer Conjugates to enhance enzyme shelf life in a liquid detergent formulation, *Macromol. Biosci.*, **2018**, *18*, 1800095.

5.4. Mesoporous Silica Nanoparticles

In this chapter mesoporous silica nanoparticles (NP) are investigated as stabilizing structure for detergent enzymes. Figure 5-23 shows the general working plan for the mesoporous silica NP.

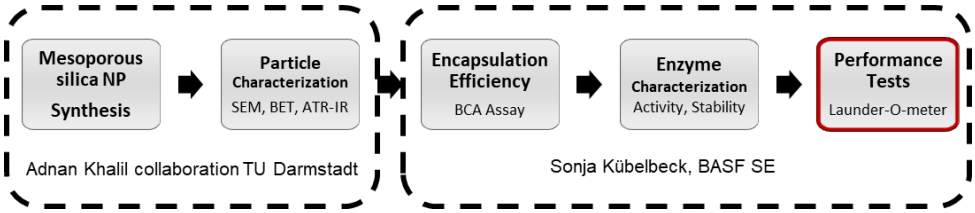


Figure 5-23: General approach for the synthesis, characterization and testing of enzyme and mesoporous silica nanoparticles.

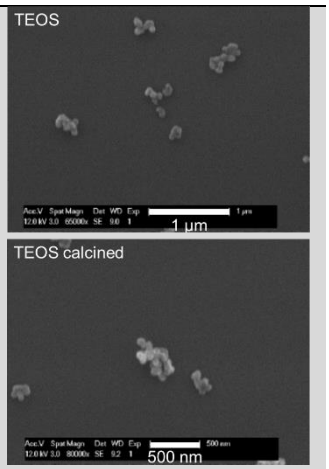
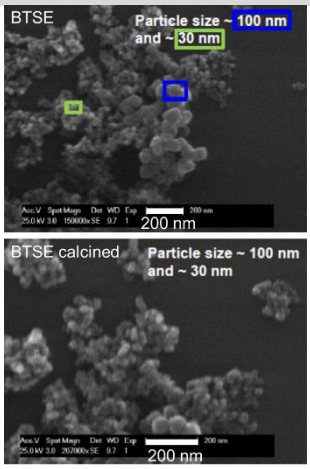
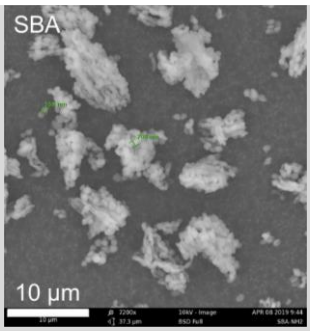
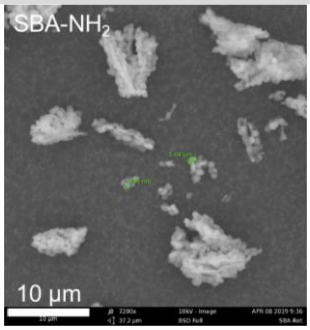
The NP are synthesized by Adnan Khalil (research group of Professor Annette Andrieu-Brunsen, TU Darmstadt) and characterized at TU Darmstadt, before the material is transferred to Ludwigshafen for the enzyme related working part (figure 5-23). Each type of the mesoporous silica NP is mixed with enzyme solutions, incubated and separated by centrifugation into supernatant and pellet. The pellet is washed and resuspended before encapsulation efficiency is determined using the BCA assay. In that context it is assumed that enzyme moieties, which is not detected in the supernatant, is immobilized within the pellet forming nanoparticles. Furthermore, the enzyme activity of supernatant as well as redispersed pellet is measured. It is envisaged that promising candidates, showing a high encapsulation efficiency and enzyme activity, will be tested in a standard detergent formulation and in the case of lipase in an aqueous solution against proteolysis. The final step is a washing test to check the enzyme performance towards standard stains on textiles (figure 5-23) At the time of submitting the thesis, no performance tests have been conducted due to the enzyme results obtained.

An overview of the silica materials synthesized by Adnan Khalil is given in the following chapter. Afterwards enzyme related experiments and results are stated. This part focuses on lipase, because so far no significant encapsulation efficiency for protease could be observed.

5.4.1. Overview of Mesoporous Silica Nanoparticles

Table 5-6 gives an overview of the mesoporous silica nanoparticles synthesized as well as their main characteristics as given by Adnan Khalil and transferred to Ludwigshafen.

Table 5-6: Overview of mesoporous silica nanoparticles synthesized/ characterized by Adnan Khalil. Pore diameters as determined by a) nitrogen sorption (Martin Brodrecht research group of Professor Gerd Buntkowsky, TU Darmstadt) or b) SEM. For original data see appendix chapter 11.3., figure 11-20 (before calcination) and figure 11-21 (after calcination).

Material	Description	Characterization	SEM image
TEOS-OTMS	TEOS, TEOS calcined	Particle size 80-100 nm Pore diameter ≈ 4-5 nm[a)]	
BTSE-OTMS	BTSE, BTSE calcined	Particle size 30-100 nm Pore diameter ≈ 5 nm[b)]	
SBA	SBA-15	Particle size 1 1.5 µm Pore diameter ≈ 8-9 nm [b)]	
SBA-NH2	SBA-15 functionalized with (3-aminopropyl)trimethoxysilane		

Mixtures of tetraethyl orthosilicate (TEOS) with octadecyltrimethoxysilane (OTMS) as well as 1,2-bis(triethoxysilyl)ethane (BTSE) with OTMS are used as silica precursors according to KALANTARI ET AL for TEOS-OTMS and BTSE-OTMA preparation.[209] Thus, the synthesized mesoporous silica NP are hydrophobic. By calcination the alkyl chains can be pyrolyzed resulting in more hydrophilic NP. Differences among the behavior in water can be observed: TEOS-OTMS particles before calcination are practically not dispersible with water and are located at the air-water interface. Due to an enhanced hydrophilicity, calcined TEOS-OTMS particles are dispersible with water. BTSE-OTMS particles are hydrophilic enough and consequently dispersible with water before and after calcination. Morphology and structure of the particles are not influenced by calcination (see SEM images in table 5-6). Success of calcination is examined by attenuated total reflection infrared spectroscopy (ATR-IR). This is possible due to the differing vibrational bands of inorganic silica and alkyl groups.[310] In preliminary tests the particle stability at alkaline pH values (pH=11) is tested for 24 h. ATR-IR spectra before and after incubation are measured and compared to identify possible differences. TEOS- and BTSE-OTMS particles before calcination are not affected by the storage conditions due to their hydrophobicity that excludes aqueous solutions. Likewise, BTSE-OTMS particles calcinated show an unaffected silica structure in SEM measurements. Slight differences regarding the Si-OH band (shoulder at 956 nm) are observed for TEOS-OTMS particles after calcination. Accordingly, incubation at alkaline pH values has only a small influence on the samples tested. Results of ATR-IR measurements are shown in the appendix chapter 11.3., figure 11-22.

Furthermore, SBA particles with a pore diameter of about 9 to 10 nm are synthesized according to GUSTAFSSON ET AL.[311] SBA particles feature a high surface area and are known to bear well-ordered mesopores with easily adjustable pore sizes. Like before, the silica material is negatively charged at basic pH values. Synthesized SBA particles are functionalized further with (3-aminopropyl)trimethoxysilane (APTMS) to introduce positively charged amino groups and to enable lipase loading at physiological pH values (lipase negatively and silica positively charged).[312]

5.4.2. Encapsulation of Lipase into Mesoporous Silica Nanoparticle

The mesoporous silica nanoparticles described before are suspended in an aqueous solution (end concentration 0.5 mg/mL) and mixed with a lipase solution (end concentration 0.5 mg/mL). For loading lipase into porous silica-based particles, the enzyme is incubated with the particles for 24 hours. Afterwards, the suspensions are separated into supernatants and pellets by centrifugation. Pellets are washed three times with water. Finally, encapsulation efficiency and lipase activity from redispersed pellets and all supernatants from washing cycles are determined by appropriate assays.

In all experiments conducted, no lipase has been detected in the supernatants of the washing cycles independent from the material or solvent tested (data not shown). TEOS-OTMS and BTSE-OTMS nanoparticles are suspended in phosphate buffer (100 mM) with pH values adjusted to 4 and 8. A pH value of 4 is below the pI of the detergent lipase and consequently, the enzyme is less negatively charged. The more alkaline detergent pH value is covered within a pH value of 8. Furthermore, SBA and SBA-NH_2 are tested in water and in a pH value series from 3 to 8. In figure 5-24 enzyme concentrations in the supernatants measured by the BCA assay are shown. It is assumed, that enzyme that is not detected in the supernatant is located in the pellet and, consequently, immobilized with the mesoporous silica nanoparticles.

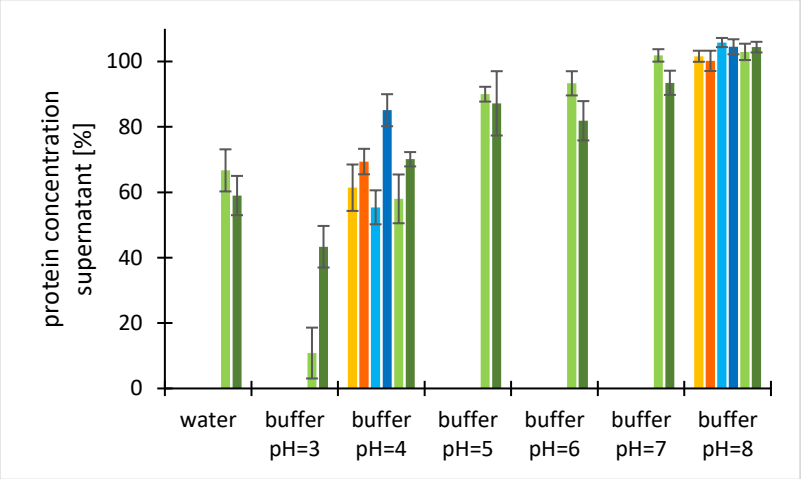


Figure 5-24: Protein concentration detected by the BCA assay in the supernatant of the incubation suspensions for lipase loading into the mesoporous silica nanoparticles: ● TEOS-OTMS ● TEOS-OTMS calcined ● BTSE-OTMS ● BTSE-OTMS calcined ● SBA ● SBA-NH_2.

As can be deducted from figure 5-24, at pH values higher than 5 almost the entire lipase is detected in the supernatant. Lower pH values lead to lower protein concentrations in the supernatants and consequently to higher encapsulation efficiencies. For instance, phosphate buffer with pH 4 shows for all materials tested an encapsulation efficiency of about 50% of the initially added lipase in solution. This equated a ratio of 500 mg lipase per gram silica. Whereas at pH 8 no lipase is encapsulated, and the complete protein concentration is measured in the supernatant. It should be noted that the detergent lipase of interest possesses an isoelectric point (pI) of about 5. As mentioned previously (chapter 2.6.3.), enzymes with pI values below 7 are more difficult to encapsulate due to electrostatic repulsion between the negatively charged enzyme and the also negatively charged silica surface (pI of about 3).[201] On this account, it has been expected that pH values below five facilitate and enable lipase encapsulation. However, the amino functionalized material, SBA-NH_2, has been expected to show an inverted pH behavior due to the present positively charged silica surface at pH values tested. Though, a substantial difference between the mesoporous silica nanoparticles tested cannot be observed

(figure 5-24). SBA functionalization occurred possibly only partially, and the main part of the silica surface is still negatively charged at pH values above 4.

Besides encapsulation efficiency, lipase activity is measured from supernatants as well as redispersed pellets. Lipase activities are measured for all conditions tested, but only results obtained at pH 4 are shown in figure 5-25. Encapsulation yields at higher pH values are low and at pH 3 lipase activity is significantly reduced due to lipase instability at these low pH value.

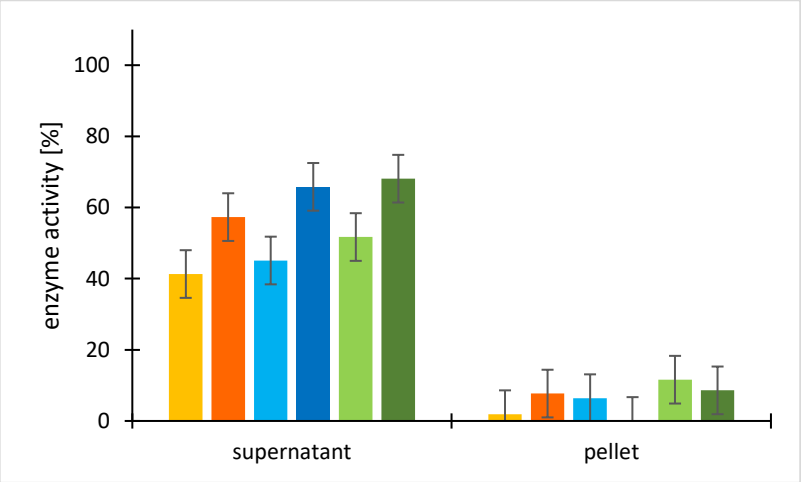


Figure 5-25: Lipase activity measured of supernatants and redispersed pellets after lipase loading in phosphate buffer at pH=4. Enzyme activity of the enzyme stock solution mixed with the nanoparticles corresponds to 100%. Shown values indicate the enzyme activities detected in supernatant and pellet. Six different mesoporous silica nanoparticles are tested. ● TEOS-OTMS ● TEOS-OTMS calcined ● BTSE-OTMS ● BTSE-OTMS calcined ● SBA ● SBA-NH_2.

Compared with the related protein concentrations of the samples (shown in figure 5-24), supernatants from SBA or SBA-NH_2 solutions retain 100% of their initial lipase activity, indicating no significant lipase denaturing by the silica materials. In the case of TEOS-OTMS and BTSE-OTMS, the measured lipase activity is lower by about 10 to 20% than expected from the protein concentration determined by the BCA assay (figure 5-25). Nevertheless, residual enzyme activities of the supernatants are reasonably equivalent with the protein contents (figure 5-25). For the pellets, a maximum enzyme activity of only 10% is measured. In order to clarify, whether the encapsulated lipase is denatured or not accessible for the substrate, a BCA assay of the redispersed pellets is conducted. Encapsulated Lipase that can be detected by the BCA assay should be accessible for 4-nitrophenyl valerate, the lipase assay substrate. In figure 5-26 protein contents in supernatant and buffer measured by the BCA assay are shown.

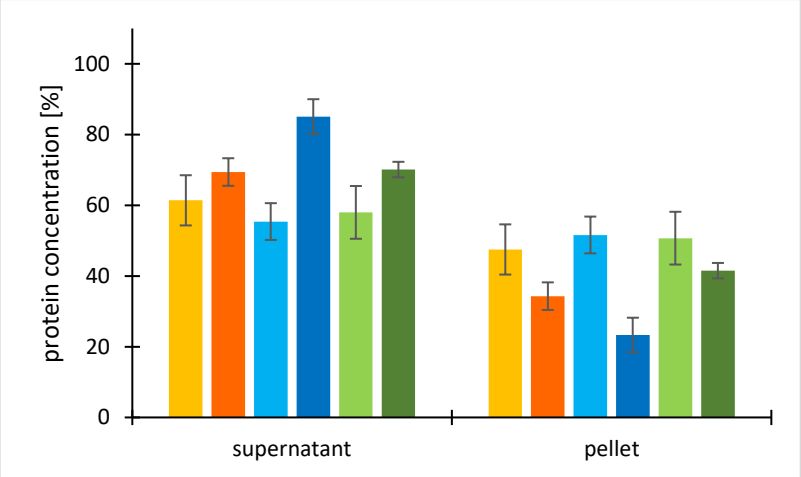


Figure 5-26: Protein concentration detected by the BCA assay in the supernatant and redispersed pellets of the incubation suspensions for lipase loading into the mesoporous silica nanoparticles in phosphate buffer at pH=4: ● TEOS-OTMS ● TEOS-OTMS calcined ● BTSE-OTMS ● BTSE-OTMS calcined ● SBA ● SBA-NH_2.

Protein concentration determined in the supernatants and in the redispersed pellets completement each other well and lipase that is not detected in the supernatant, is measured in the corresponding pellet (figure 5-26). Accordingly, it must be assumed, that large parts of the encapsulated lipase are denatured upon encapsulation into the silica nanoparticles. In general, lipase is stable under the terms of immobilization as shown in chapter 5.1.1. The reason for the loss of lipase activity is unlikely the presence of charge on the silica surface due to existing experience with lipase and polyelectrolytes and charged materials in other BASF projects. It is more likely that the pH value inside the pores differ from the surrounding buffer pH value. A high proton content might be confined in the pores and lipase molecules that diffuse in such pores are exposed to denaturing conditions. In this connection, the group of IGAL SZLEIFER observed charged properties, depending on the nanochannel geometry, that differ from the expected bulk materials.[313] A significant stability difference between pH 3 and 4 (see figure 5-1) occurs for lipase. Appropriate, the low lipase activities can be a result of a lower pH value in the pore.

Due to the low lipase activities measured for encapsulated lipase, no further experiments – storage tests in a standard detergent formulation or washing experiments – are conducted.

There are many examples of a successful enzyme encapsulation into mesoporous silica nanoparticles in literature. For instance, the synthesis of TEOS-OTMS particles is in accordance with KALANTARI ET AL., who immobilized lipase from *candida rugosa* successfully.[209] They observed high lipase loadings at pH values higher than the enzymes´ isoelectric point despite the expected electrostatic repulsion. Additionally, an increased lipase activity is measured for all particles tested. The increased relative activity is explained by the hydrophobic environment that promotes an open lid of the lipase active site. This hypothesis is supported by an observed decreased relative activity if calcinated – more

hydrophilic – nanoparticles are used for lipase immobilization. The observations by KALANTARI ET AL. are not consistent to my observations made within this thesis. It is possible that the detergent lipase is more vulnerable to an extreme hydrophobic environment than lipase from *candida rugosa* that is engineered for applications in the biocatalysis and for organic solvent exposure. Low encapsulation efficiencies (25%) but increased relative lipase activities are observed by GUSTAFSSON ET AL.[311] In this case, large differences in the assay implementation are present. GUSTAFSSON ET AL. incubate immobilized lipase and substrate solution for 1.5 h before the release of 4-nitrophenol is measured spectroscopically. The standardized lipase activity assay used in the present thesis operates with an incubation time of 2 minutes. Accordingly, it is possible that in the case of mesoporous silica nanoparticles higher incubation times are necessary to capture all active and encapsulated enzyme. For a successful lipase assay, 4-nitrophenyl valerate, a small and relatively hydrophobic molecule must diffuse into the pores. Nevertheless, these results represent an interesting starting point for further systematic investigations of confinement effect influences on enzyme stability.

Chapter 5.4. can be summarized as follows: six different mesoporous silica nanoparticles haven been synthesized successfully by Adnan Khalil. Those materials are characterized by ATR-IR, SEM and BET regarding particle and pore size. The last-mentioned pore size with 5 to 10 nm are large enough to encapsulate lipase (3 nm). It has been observed that a pH value of 4 leads to highest encapsulation efficiencies of 50% at best. However, residual enzyme activities of encapsulated lipase are significantly reduced (10%). It can be assumed that the enzyme is denatured by low pH values present in the silica pores and that spatial confinement plays a crucial role for enzyme stability.

5.5. Metal-Organic Frameworks

This chapter deals with metal-organic frameworks (MOFs) as enzyme stabilizing agent in a liquid detergent. The general approach is displayed in figure 5-27 starting with co-precipitation for enzyme immobilization and encapsulation (enzyme ⊂ MOF). As described in chapter 2.7.1. co-precipitation is used to combine enzymes with MOFs due to the unsatisfied results obtained with presynthesized mesoporous silica nanoparticles. Enzyme and MOF precursors co-precipitate and the framework is built up around the enzyme *in-situ* (figure 5-27). After three washing cycles of the MOF-enzyme adduct, the MOF is characterized regarding crystallinity (PXRD) and crystal structure (TEM). Encapsulation efficiency is determined – if possible – using the BCA assay of the supernatant removed in the first washing cycle. It is assumed that enzyme molecules, which are not recovered in the supernatant are encapsulated in or immobilized onto the MOF. The enzyme activity of the redispersed washed MOF phase is measured to calculate the amount of active enzyme within the MOF. MOFs with promising results regarding encapsulation efficiency and enzyme activity are examined in storage tests in standard detergent formulations and in the case of lipase in aqueous solutions against protease degradation. Finally, lead candidates are investigated in washing tests whether the encapsulation or immobilization using MOFs has an influence on the enzymes´ performance towards standard stains on textiles (figure 5-27).

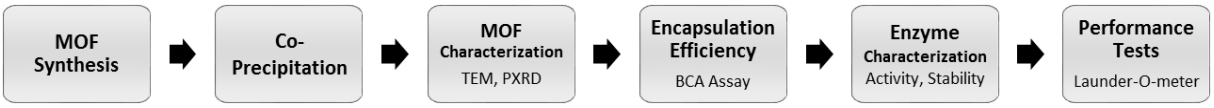


Figure 5-27: General approach for the synthesis, characterization and testing of enzyme ⊂ MOF.

In the following chapters the characterization results using co-precipitation are reported. Afterwards lead candidates undergo storage and performance tests.

5.5.1. Synthesis of Enzyme ⊂ MOF

Preliminary tests (data not shown) demonstrated that lipase is most suitable for co-precipitation. For example, protease is rather unsuitable due to the high pI for co-precipitation and formation of ZIF-8. MADDIGAN ET AL. showed that the MOF formation is compromised if the pI of the protein is higher than 7 as given for the protease.[314] The pI of lipase is sufficiently low (about 5.0) and the enzyme is robust towards organic compounds. On this account, the combination of MOFs and enzymes is focused on lipase. Thereby, ZIF-8, MOF-74 and MIL-53 as frameworks are tested and the results are presented in the following subsections.

Lipase ⊂ ZIF-8

To achieve lipase encapsulation in ZIF-8, the enzyme is mixed with 2-methylimidazole (HmIm) and stirred constantly during and after the addition of zinc nitrate hexahydrate. A solid material is formed at the latest overnight. The washed pellet is studied by TEM and PXRD. Not every precursor combination lead to the formation of a crystalline MOF. TEM and PXRD results display that the ratio of precursors determines pattern and crystal size (figure 5-28). LO ET AL. report the phases HmIm and Zn^{2+} can form.[315] According to their study, it is possible to identify phases and to create a phase diagram. In the diagram shown in figure 5-28 the enzyme concentration is held constant.

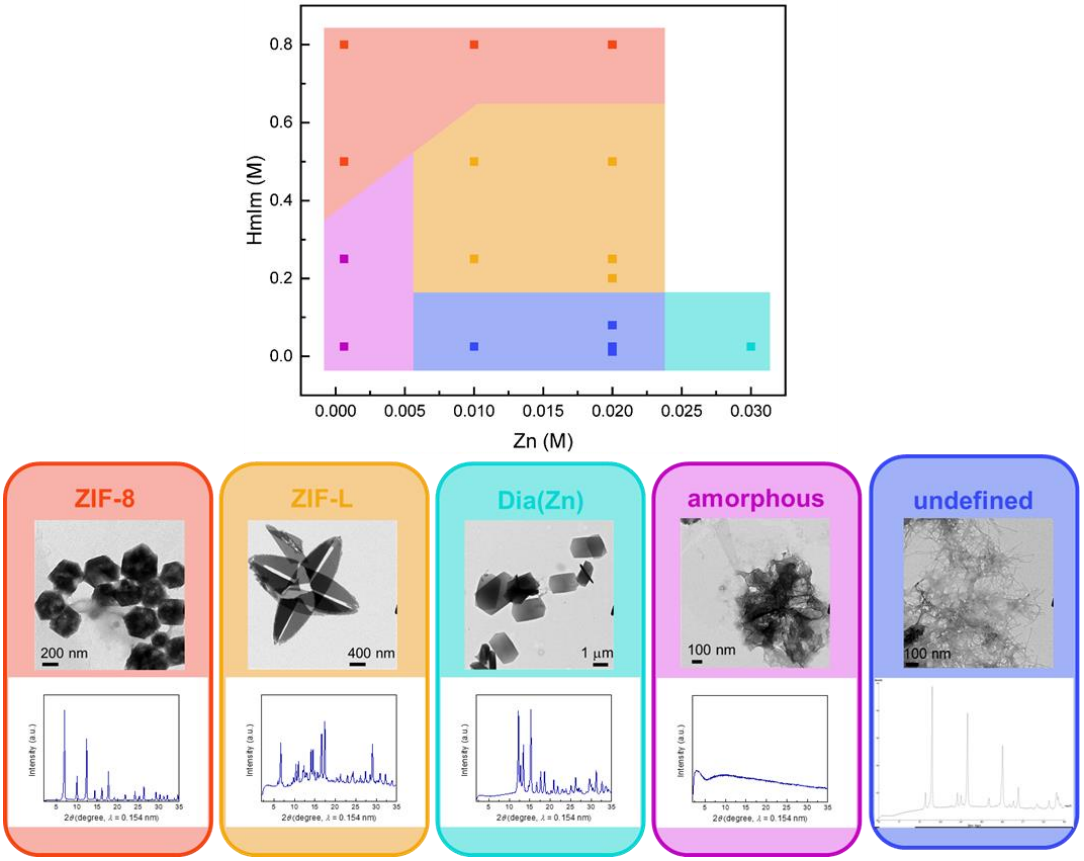


Figure 5-28: Phase diagram of ZIF-8 and lipase. The formation of ZIF-8, ZIF-L, dia(Zn), amorphous and undefined material can be observed as indicated by the color code. Scattering data are analyzed by Zhe Ji (UC Berkeley).

In general, four different polymorphs formed with zinc(II) and 2-methylimidazole are known based on present knowledge. Best known is ZIF-8, build up by the coordination of the zinc ions by four imidazole rings forms rhombic dodecahedron crystals (figure 5-28). ZIF-L, first synthesized in 2013,[316] shows a leaf-like structure with a layered topology in TEM images.[315] A diamondoid crystal topology possess dia(Zn),[317] which has been synthesized firstly 2011.[318] Formation of this polymorph has not been expected, due to the normally required elevated temperatures (60 °C) and the presence of a

catalyst.[315] These results indicate that lipase can act as an catalyst and lower the synthesis temperature for the formation of dia(Zn).

The amorphous and undefined phase differ from the other phases. Under conditions forming amorphous and undefined phases, no structure (amorphous) and a worm like structure (undefined) can be observed (figure 5-28). The occurrence of the latter phase is not described in literature. To the best of my knowledge, the undefined phase is described for the first time.

Further studies show that independent of the formed phase very high encapsulation efficiencies – determined by BCA assay – can be obtained. In this way it has been possible to encapsulate the entire used enzyme into the constructed framework. The residual activity of embedded lipase is measured after redispersing the solid. Here, a maximum of 50% lipase is sufficiently active to degrade the assay substrate and to release the detected dye. Encapsulation yield is the product of encapsulation efficiency and residual activity of embedded lipase. In figure 5-29 the encapsulation yields of lipase and ZIF-8 is shown.

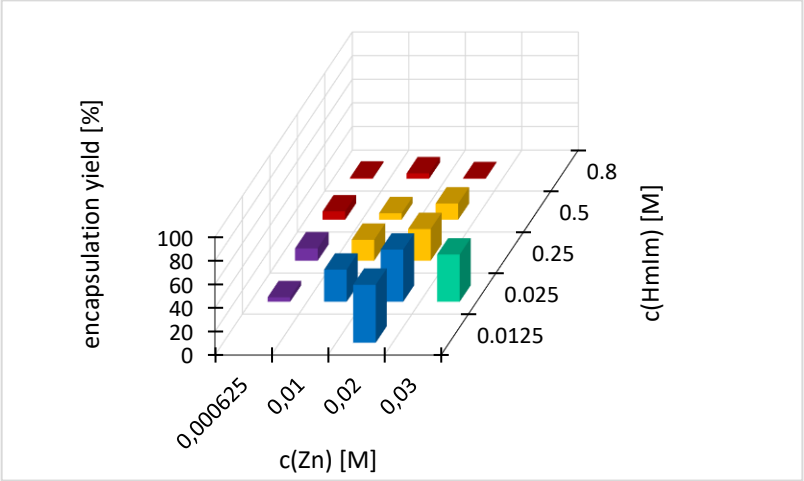


Figure 5-29: Encapsulation yield of lipase in ZIF-8. The yield is a product of encapsulation efficiency (mostly 100%) and residual activity of embedded lipase (less than 50%). The colors symbolize the different phases identified before. The average error for encapsulation yields is between 2 and 8%. ● ZIF-8 ● ZIF-L ● dia(Zn) ● amorphous ● undefined.

As illustrated in figure 5-29, the formation of ZIF-8 and ZIF-L leads to an almost complete inactivation of lipase and consequently to a low encapsulation yield. A similar behavior can be observed for the amorphous material: the encapsulation efficiency and residual lipase activity are low. Highest encapsulation yields – up to 50% – are obtained for dia(Zn) and the undefined phase. Reffering to literature,[315] it has been expected that the microporosity of ZIF-8 has a positive effect on the accessibility of substrate to lipase. However, if ZIF-8 (figure 5-29 red) is formed no enzyme activity can be measured. It is possible that the tertiary and secondary structure of lipase is destroyed upon crystal formation. Interestingly, lipase activity can be measured if the MOF is present as dia(Zn), which is

expected to be almost nonporous.[315] The porosity of the surrounding framework appears less important for the residual lipase activity.

Due to the low lipase encapsulation yields within ZIF-8 and ZIF-L as well as the amorphous and the dia(Zn) phase, it has been focused on the undefined phase for further experiments.

Lipase ⊂ MOF-74

Equal to ZIF-8, lipase is mixed with the organic linker – 2,5-dihydroxyterephthalic acid (DHTP) – and zinc nitrate hexahydrate is added under continuous stirring. In order to let the MOF formation take place in aqueous solution, DHTP is transferred into its water-soluble disodium salt using sodium hydroxide. Studies using TEM and PXRD show that the pH value of the DHTP solution is crucial for successful MOF formation. At a pH value below 10 a crystalline pattern can be observed, while higher pH values lead to an amorphous solid. In figure 5-30 images of appropriate TEM and PXRD measurements are shown.

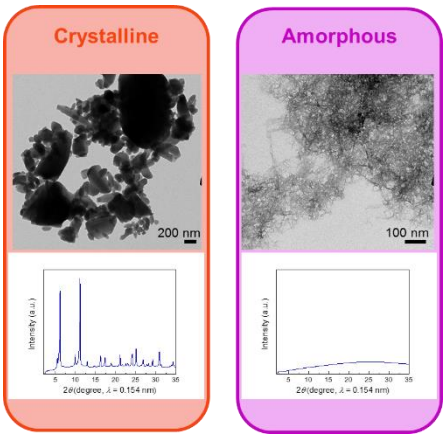


Figure 5-30: TEM and PXRD measurement of MOF-74 synthesis. A pH value up to 10 leads to the formation of a crystalline MOF-74 (left ●). If the pH value is higher than 10, an amorphous solid is formed (right ●). Scattering data are analyzed by Zhe Ji (UC Berkeley).

Both – crystalline and amorphous – solid materials are used to calculate residual enzyme activity and the encapsulation yield. The chelating effect of DHTP makes it impossible to perform a BCA assay of the supernatant for determination of encapsulation efficiency. However, lipase activity is measured from redispersed pellets and supernatants after centrifugation. In that case, the residual enzyme activity in the pellet corresponds to the encapsulation yield. Independent of the present MOF structure, no enzyme activity can be detected in the supernatant. Accordingly, high lipase activities can be determined for amorphous solid materials (between 80 and 90%). Crystalline MOF-74 performs worse than the amorphous one and measured encapsulation yields are reduced to 40%. Figure 5-31

shows the direct comparison of crystalline and amorphous MOF-74 using same precursor concentration with a different pH value of the DHTP solution.

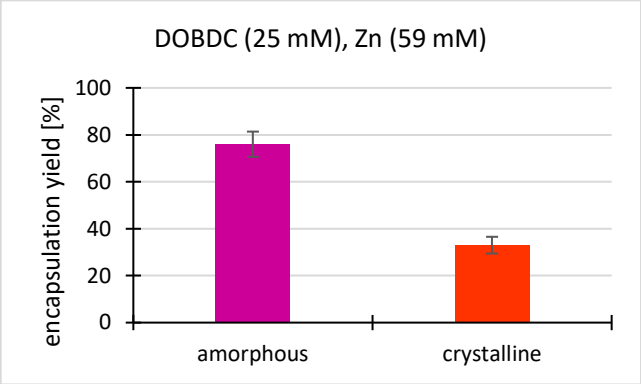


Figure 5-31: Encapsulation yield of amorphous ● and crystalline ● pattern using the same precursor concentrations and different pH values of DHTP solutions.

In general, the inhomogeneous crystals of MOF-74 have a large size (> 500 nm; figure 5-31) which might destroy the structure of lipase resulting in a significantly decreased enzyme activity. Nevertheless, in further experiments amorphous and crystalline materials are used for storage tests.

Lipase ⊂ MIL-53

The synthesis of MIL-53 is carried out in aqueous solution and lipase is mixed with terephthalic acid before aluminum nitrate nonahydrate is added. Terephthalic acid is used as a disodium salt formed by the addition of sodium hydroxide. The pH value of the linker solution determines the pattern as for MOF-74. The pH 10 threshold defines if an amorphous or a crystalline material is formed. TEM and PXRD images are shown in figure 5-32.

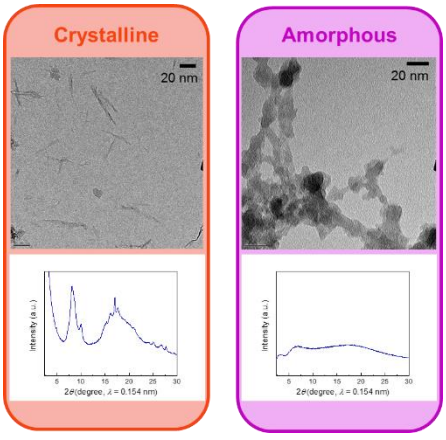


Figure 5-32: TEM and PXRD measurement of MIL-53 synthesis. A pH value up to 10 leads to the formation of a crystalline MIL-53 (left ●). If the pH value is higher than 10, an amorphous solid is formed (right ●). Scattering data are analyzed by Zhe Ji (UC Berkeley).

Crystalline MIL-53 possesses thin crystals with a worm-like structure – few nm thick and up to 50 nm long. The amorphous material is not processed further, and encapsulation yield is determined only for the crystalline pattern. Here, several ratios of terephthalic acid and aluminum nitrate with a constant lipase concentration are tested and the residual enzyme activity of supernatants and redispersed pellets after centrifugation is measured. Copper complexing properties of terephthalic acid disables the implementation of a BCA assay for determination of encapsulation efficiency. The residual enzyme activity is equal to the encapsulation yield. Encapsulation yields of lipase in MIL-53 are illustrated in figure 5-33.

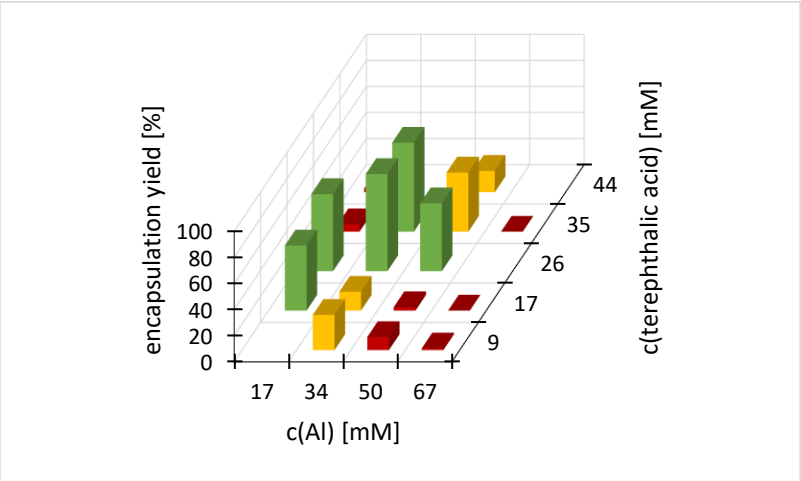


Figure 5-33: Encapsulation yield of lipase into crystalline MIL-53 with various ratios of terephthalic acid and aluminum nitrate. Encapsulation yields lower than 10% (●), between 10 and 50% (●) and higher than 50% (●). The average error for encapsulation yields is between 2 and 8%.

No lipase activity can be measured in the supernatant of MIL-53 independently of the applied precursor ratio. A wide variance regarding residual lipase activity in the redispersed pellet is observed (figure 5-33). Equimolar ratios of terephthalic acid and aluminum nitrate seem to enable the highest encapsulations yields for lipase (up to 75%). These precursor concentrations are used for further experiments.

In figure 5-34 summarizes the encapsulation yields of the MOFs used for storage tests and further characterizations. Highest encapsulation yields are obtained for amorphous “MOF-74” and crystalline MIL-53 (83% and 75% respectively). Using the undefined ZIF-8 pattern or crystalline MOF-74, encapsulation yields less than 50% are measured.

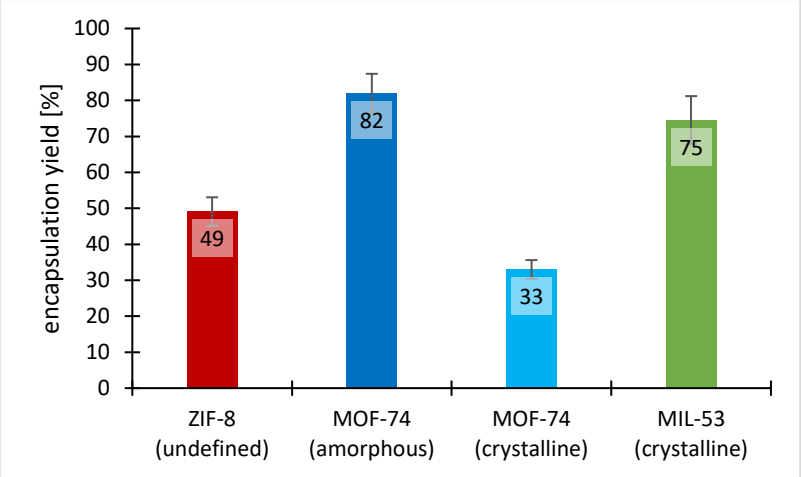


Figure 5-34: Encapsulation yields of the lead candidates. ● Undefined ZIF-8 phase, ● amorphous MOF-74, ● crystalline MOF-74, ● crystalline MIL-53.

5.5.2. Testing of Enzyme ⊂ MOF

As described in the previous chapter (5.5.1.), four different MOFs have been identified to encapsulate the highest amount of lipase while the enzyme maintains its activity. It refers to the undefined ZIF-8 pattern, amorphous and crystalline MOF-74 as well as crystalline MIL-53. These lead candidates are tested against a standard detergent formulation and against proteolysis. In further experiments washing performance tests against stained textiles are conducted to check if lipase embedded into a metal-organic framework can still perform.

Results of storage tests of the four lead candidates with embedded lipase in a standard detergent formulation and in water containing protease are shown in figure 5-35. Solutions for experiments in SDF are stored at 37 °C, tests against protease at 22 °C.

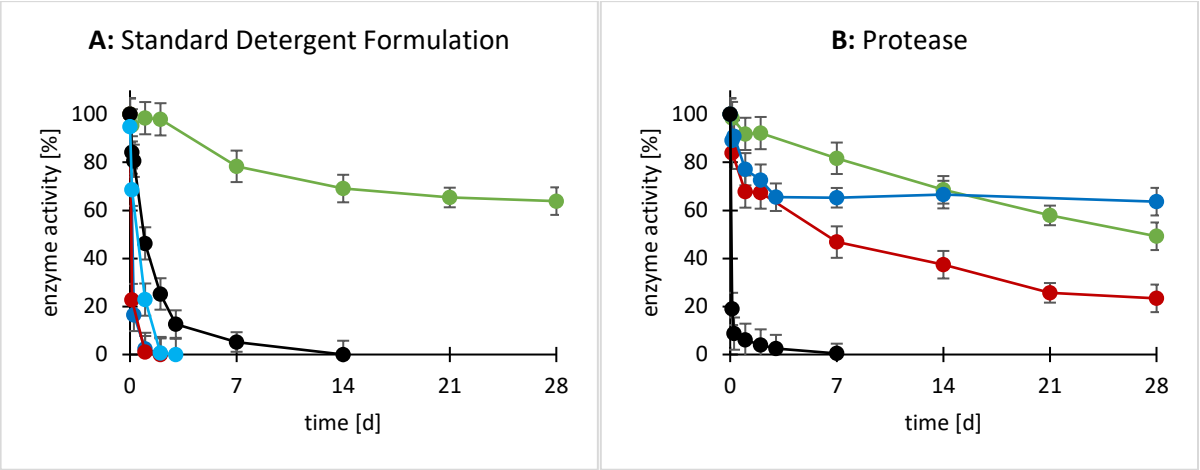


Figure 5-35: Graphical representation of the storage tests of lipase embedded into MOFs in a standard detergent formulation at 37 °C (A) and in water against protease at room temperature (B). ● Undefined ZIF-8 phase, ● amorphous MOF-74, ● crystalline MOF-74, ● crystalline MIL-53 ● pure lipase. Connecting lines are only guide to the eye.

Pure lipase stored in an SDF at 37 °C loses its activity completely after two weeks (figure 5-35 A). Assembly of a metal-organic framework around the enzyme shows no stabilization effect in case of ZIF-8 and crystalline as well as amorphous MOF-74 (no enzyme activity after three days). It even seems that the enzyme loses its performance even faster than without any protection. On the other hand, embedding lipase into MIL-53 leads to a significant increase in enzyme stability. After two weeks about 70% and after four weeks more than 60% retain their activity.

In an aqueous solution containing protease, the pure lipase is completely inactive after three days (figure 5-35 B). Under these conditions, all three MOFs, ZIF-8, MOF-74 and MIL-53, can increase the stability of lipase significantly. Embedded into ZIF-8, about 20% of lipase is active after four weeks storage. The stabilizing effect of MIL-53 leads to an enzyme activity of about 50% after four weeks. MOF-74 improves the stabilization of lipase further and after four weeks more than 60% of lipase remains active. As described for the other stabilizing systems (small molecules, enzyme-polymer conjugates and mesoporous silica nanoparticles), the protease stability is studied as well. The protease stability is not influenced by the MOFs and appropriate diagrams are shown in the appendix (chapter 11.5., figure 11-23).

It should be mentioned that the addition of ZIF-8 and MOF-74 to an SDF solution results in turbid colloidal unstable solutions. Whereas the addition of MIL-53 leads to a clear and stable solution (figure 5-36). In regard to a sales product, the transparent product is clearly preferred for liquid detergents.

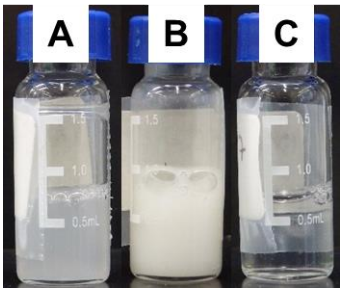


Figure 5-36: Image of the storage solutions in an SDF. A: ZIF-8, B: MOF-74, C: MIL-53.

MIL-53 and MOF-74 have a very similar linker – terephthalic acid and 2,5-dihydroxyterephthalic acid – and show clear differences in stability. Using MOF-74, lipase shows a decreased stability in SDF, whereas the stability against proteolysis in water is increased. In contrast, it is possible to enhance the stability of lipase in SDF and against proteolysis if the enzyme is embedded into MIL-53. One explanation for this behavior is that trivalent metal species can offer an enhanced stability to MOFs in aqueous solutions.[239] MOF-74 and ZIF-8 are formed with a bivalent metal species (Zn^{2+}), whereas MIL-53 contains a trivalent aluminum (Al^{3+}). The high ion strength that is present in SDF represents a challenge for metal-organic frameworks and might lead to a destruction of the two bivalent metal MOFs (ZIF-8 and MOF-74), while MIL-53 with the trivalent metal species remains stable. In an aqueous solution with a low ion strength, the bivalent MOFs are stable as well and can protect lipase against degradation by protease. Here, MOF-74 performs significantly better than MIL-53 so that the residual enzyme activity after two weeks is about three times higher for lipase embedded into MOF-74. It is possible that the twofold substituted terephthalic acid in case of MOF-74 shows an improved shielding from protease compared to the unsubstituted terephthalic acid of MIL-53. A leaching effect of should be rather likely. Lipase concentration of a washed sample with MIL-53 has been measured after several days of storage and no lipase has been detected in the supernatant (data not shown).

Due to the risk of a steric blockade of the lipase active site by the MOF formation around the enzyme, performance tests are necessary to prove that the active site is still accessible for real stain removal on textile fibers. On this account, lipase embedded within MIL-53 is selected and tested in a performance test. Three different concentrations of pure lipase as well as incorporated lipase are washed in a launder-o-meter at 25 °C together with clothes soiled with fatty stains residues for 30 minutes. The stains include, inter alia, lard. For analysis, the L* value is measured by a photometer before and after the washing process. The brightness of the textile – the washing success – is quantified and resulting dL* values are plotted against the enzyme concentration (ppm) in figure 5-37.

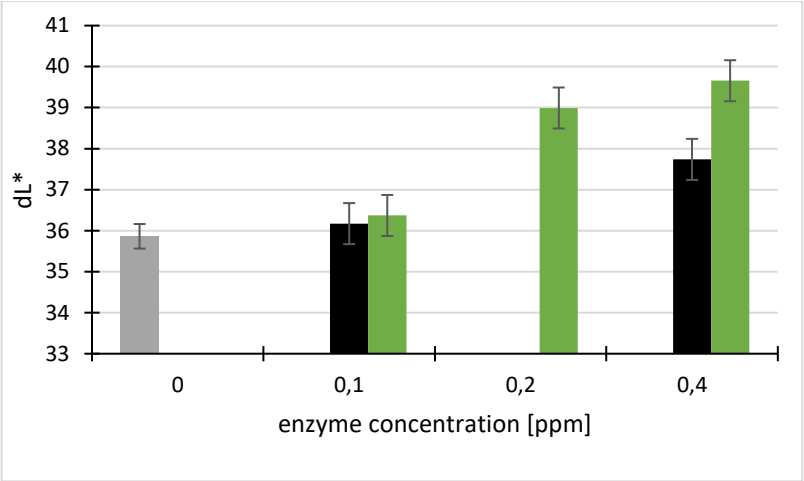


Figure 5-37: Washing performance of pure lipase and lipase ⊂ MIL-53. Washing tests are performed in a liquid standard detergent formulation at 25 °C with three different enzyme concentrations (0.1, 0,2 and 0,4 ppm; pure lipase 0,2 ppm is not tested). As a reference, the washing effect of the pure detergent is shown (●). ● pure enzyme ● lipase ⊂ MIL-53.

The median concentration of pure lipase is not tested. As figure 5-37 shows, lipase embedded into MIL-53 is at least as active as the pure lipase. Lipase activity towards large fatty stains is not reduced by MOF formation. There is even the option that the embedded lipase shows a better performance than the free lipase. One reason for this could be the protective effect of the MOF whereas the pure lipase is exposed to the surfactants of the detergent formulation.

In summary, three different metal-organic frameworks (ZIF-8, MOF-74, MIL-53) have been synthesized *in-situ* around lipase. The formation of ZIF-8 in combination with enzymes is well described in literature, whereas novel synthesis routes for the two other MOFs have been used. Depending on precursor ratio (ZIF-8) or on pH value (MOF-74, MIL-53), different morphologies have been synthesized. These morphologies influence the encapsulation yield of lipase: high encapsulation yields have been obtained for all MOFs. The critical aspect is the residual enzyme activity after encapsulation. Highest encapsulation yields haven been observed with amorphous MOF-74 (82%) and MIL-53 (75%). Lower yields are achieved with undefined ZIF-8 (49%) and crystalline MOF-74 (33%).

Storage tests against an SDF showed that MIL-53 significantly increases lipase stability, whereas the other MOFs do not show a stabilizing effect on the lipase (figure 5-35 A). In a storage test in water against protease, all MOFs tested enhance lipase stability – particularly well MOF-74. Regarding an application in a liquid detergent, a performance test shows that lipase embedded into MIL-53 has at least the same cleaning power as the free enzyme.

In a nutshell, MOF synthesis via co-precipitation represents an effective innovative method for enzyme stabilization in a liquid detergent formulation.

The work shown in this chapter has been conducted mainly at UC Berkeley during my stay in Omar Yaghis group. Zhe Ji introduced me into the MOF topic. He instructed and supported TEM and PXRD measurements, especially analysis of scattering data. Enzyme-related measurements (encapsulation yield, thermal stability, storage tests) have been performed without assistance. Claudia Esper (BASF SE) is responsible for the washing performance tests. Parts of this work will be published, after intellectual property (IP) will have been generated.

6. Summary and Conclusion

The aim of this work is the development and comparison of four different enzyme stabilizing systems for liquid detergent application. Thereby, the systems pursue different strategies to enhance enzyme stabilization. Enzymes of interest in detergent applications include a protease, an α-amylase and a lipase. In contrast to the state-of-the-art, stabilization is not limited to protease inhibition. Here, a special focus is on lipase – an enzyme that is vulnerable to proteolysis. This and the high liability of all three enzymes in a standard liquid detergent formulation has been demonstrated firstly. Generally, two independent methods for enzyme stability determination are used: enzyme activity in storage tests and thermal stability in nanoDSC experiments. As one key result, a good correlation between both methods has been identified. This correlation enables a long-term enzyme activity prediction based on one thermal stability measurement. Consequently, the number of time-consuming long-term storage tests can be reduced in future.

Starting with **small molecules** as stabilizing agents, four related ester compounds are used to enhance lipase stability. All four molecules tested target lipase and increase the lipase stability. This has been demonstrated by an enhanced thermal stability in nanoDSC measurements as well as in storage tests at elevated temperatures. Both experiments are conducted in a standard liquid detergent formulation. As expected, the residual lipase activity is not reduced by the presence of the small molecules. Measurements in a standard detergent formulation reveals that lipase can be stabilized by small molecules up to four weeks at elevated temperatures. Although, lipase stabilization against proteolysis has not been anticipated, lipase stability towards proteolysis increased significantly in the presence of choline-based small molecules. However, the effect can be attributed to a protease denaturing due to the choline compounds. Such an irreversible protease inactivation is undesired. Even if the denaturing of protease occurs only in water and not in a standard detergent formulation (SDF), other enzymes like α-amylase are influenced negatively with respect to their stability by at least one of the small molecules tested. It has been learned that a molecule that stabilizes one enzyme can act as an inhibitor or inactivator for another enzyme. For this reason, in complex enzyme mixtures, like detergent formulations, all enzymes must be taken into account to identify stabilizing small molecules.

Summary:

- Formulation difficulties: No (possible problems with water solubility of the small molecules)
- Stability in a liquid detergent formulation and against proteolysis: Lipase stability in an SDF and against proteolysis significantly increased in a long-term.
- Answers to open questions:
    - Targeting of lipase possible? Yes.

- Influence on other enzymes (protease and α amylase)? Yes, protease and α amylase show under certain conditions a reduced activity.

It has been noted, that the success of the **enzyme-polymer conjugate** synthesis depends greatly on the enzyme used. Due to a higher number of accessible lysine residues, conjugation with α-amylase and lipase is achieved easily and completely for all polymers used (mPEG-aldehyde, mPEG-NHS, maltodextrin-aldehyde, CMC-aldehyde). On the contrary, protease conjugation has been incomplete for the activated PEGs. In general, conjugation is achieved more easily with the oxidized polysaccharides than with the functional PEGs. Presumed cause might be the availability of more than one attachment point per polymer molecule. Furthermore, regarding stability experiments, the oxidized polysaccharides performed better in case of protease and α-amylase. The more hydrophobic lipase is more stable in presence of PEG. Because of the washing results, which demonstrate retained performance for α-amylase conjugated to maltodextrin in contrast to the PEG conjugated one, polysaccharides are the better choice for conjugation of detergent enzymes.
It has been expected that the residual enzyme activity is reduced by the conjugation of the enzyme. In fact, conjugated enzymes retain their activity in large parts, so that in most cases more than 80% are still active. Shelf-life of protease, α-amylase and lipase in a liquid standard detergent formulation and against proteolysis in the case of lipase has been increased due to the conjugation to polymers. Nevertheless, observed stabilization effects are short-term and lower than anticipated.
Summary:

- Formulation difficulties: Dependence on accessible lysine residues (low conversion rate for protease).
- Stability in a liquid detergent formulation and against proteolysis: Short-term stabilization effects for conjugated protease, α amylase and lipase in an SDF and for lipase against proteolysis.
- Answers to open questions:
    - Glycosylation or PEGylation? Glycosylation performs and protects in general better.
    - Accessibility of large substrates (stain) given? Glycosylated α-amylase retains performance, PEGylated one is worse.

Lipase loading into pre-synthesized **mesoporous silica nanoparticles** while retaining lipase activity has proven to be difficult. It has been possible to immobilize about 50% of the initial lipase solution (500 mg lipase per g silica). Considering the electrostatic repulsion due to identical charges of enzyme and silica surface, the encapsulation efficiency is sufficient. However, the encapsulated lipase loses its activity almost completely. On this account no further experiments regarding the application are conducted.

Nevertheless, the measurements indicate that a confinement effect on pH is responsible for the loss of lipase activity by immobilization within the pores. Further systematic investigations have to analyze confinement influences on enzyme stability. The strategy of using pre-synthesized carrier material, which requires enzyme diffusion into the material is laid aside for detergent applications.

Summary:

- Formulation difficulties: Great losses of enzyme activity due to lipase immobilization.
- Stability in a liquid detergent formulation and against proteolysis: Not tested.
- Open research questions cannot be answered due to the too low residual lipase activity after immobilization.

In the case of the **metal-organic frameworks**, lipase is encapsulated by the *in-situ* formation of the carrier material around the enzyme. High encapsulation yields (100%) are achieved easily and by tuning the precursor ratio it is possible to reduce the lipase activity loss. Total encapsulation yields of 33 to more than 80% are obtained. Three different MOFs – ZIF-8, MOF-74 and MIL-53 – have been tested and compared. Regarding lipase protection in a standard liquid detergent formulation, MIL-53 increases the lipase stability significantly up to more than 60% compared with the complete inactive pure lipase after four weeks, while ZIF-8 and MOF-74 show a destabilizing effect. It can be assumed that ZIF-8 and MOF-74, as MOFs formed with a bivalent metal species (Zn^{2+}), are unstable within an SDF. Expected stabilizing effects regarding proteolysis are observed for all three MOFs tested. Performance tests with lipase encapsulated in MIL-53 show no reduction of cleaning power compared to the free enzyme. Therefore and because of the stability and stabilization effect in an SDF, MIL-53 is the most promising MOF for detergent applications.

Summary:

- Formulation difficulties: No.
- Stability in a liquid detergent formulation and against proteolysis: Lipase stability in an SDF and against proteolysis significantly increased in a long-term using MIL 53.
- Answers to open questions:
    - MOF stability in a liquid detergent? Yes, MOFs with trivalent metal species are stable.
    - ZIF-8, MOF-74 or MIL-53? MIL-53 protects and performs best.
    - Accessibility of large substrates (stain) given? Yes, lipase in MIL-53 performs as good as pure lipase.

In figure 6-1 the lead candidates for lipase stabilization in liquid detergent formulations identified by the stabilization strategies tested are shown. Due to the different lipase batches and differences between the lipases used, the enzyme activities are plotted separately. In the case of small molecules

(A and B), citric acid choline ester (CACE) is chosen as lead candidate. For the enzyme-polymer conjugates (C and D) it is mPEG-NHS and for the metal-organic frameworks (E and F) MIL-53. Storage in an SDF (left) and in water against proteolysis (right) are compared with the pure appropriate lipase.

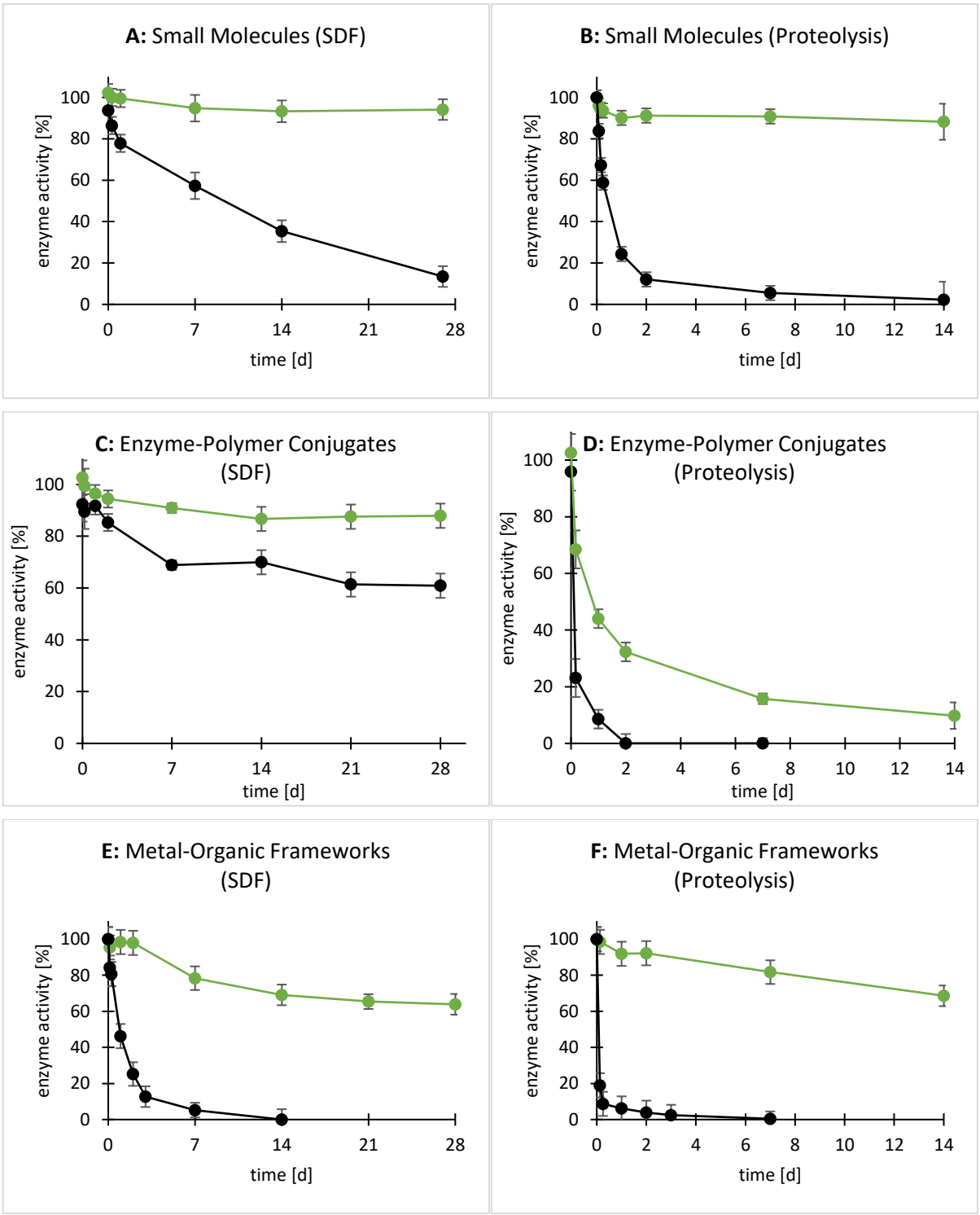


Figure 6-1: Overview of the lead candidates for lipase identified for each stabilization strategy. Pure lipase and stabilized lipase are stored in a standard detergent formulation at 37 °C (C: 22 °C) and in water with the presence of protease at 22 °C. A+B: small molecules, CACE; C+D: enzyme-polymer conjugates, mPEG-NHS, E+F: metal-organic frameworks, MIL-53. ● lead candidate ● pure lipase. Connecting lines are only guide to the eye.

It should be noted that the storage test with the enzyme-polymer conjugate has been conducted at 22 °C, whereas the tests with the small molecules and the MOFs are performed at 37 °C. By comparing the lead candidates, the most significant effect on the lipase stability is obtained by the addition of the small molecule CACE (figure 6-1). Almost the complete lipase activity is retained in a standard detergent formulation at elevated temperatures as well as in presence of protease at room temperature for up to four weeks (figure 6-1 A+B). Under the tested conditions, this system accomplishes the targets imposed. Nevertheless, further experiments in the presence of all detergent enzymes are necessary to confirm the high lipase stability observed on the one hand and to examine the influence on CACE on other enzymes on the other hand. In this regard, stabilization by the *in-situ* formation of metal-organic frameworks is less critical. Stabilization results obtained for MIL-53 are promising and expandable for example by using different organic linkers in order to prevent leaching (figure 6-1 E+F). Enzyme-polymer conjugates perform worst (mesoporous silica nanoparticles excepted) and the lipase stability is increased only in a short-term (figure 6-1 C+D).

With respect to industrial frameworks, small molecules are the most attractive method for enzyme stabilization in liquid detergents. It is finally noted that no additional synthesis or purification step is necessary, and the risk of an enzyme performance loss is unlikely. However, the identification and development of enzyme specific stabilizing small molecules is an elaborate process. The other strategies are more universal and transferable between the different enzyme classes. It is easier to develop a precursor ratio to form a stabilizing MOF around an enzyme than screening for a selective small molecule stabilizer. On this account, metal-organic frameworks seems to be a very promising completely new alternative to the established small molecules for enzyme stabilization in liquid detergent formulations.

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8. List of Abbreviations

A	Alanine
AC	Acetylcholine
AChE	Acetylcholinesterase
AF4	Asymmetric flow field flow fractionation
Ala	Alanine
Ald	Aldehyde
1,8-ANS	8-anilinonaphthalene-1-sulfonic acid
APTMS	3-Aminopropyltrimethoxysilane
Asp	Asparagine
ATEC	Acetyl triethyl citrate
ATR-IR	attenuated total reflection infrared spectroscopy
BCA	Bicinchoninic acid
BTSE	1,2 bis(triethoxysilyl)ethane
CACE	Citric acid choline ester
CLEAS	Cross-linked enzymes aggregates
CMC	Carboxymethyl cellulose
COF	Covalent-organic framework
CTAB	Cetyltrimethylammonium bromide
1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
DAD	Diode array detector
DCC	Dicyclohexylcarbodiimide
DHTP	2,5-dihydroxyterephthalic acid
DLS	Dynamic light scattering
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DoE	Design of Experiment
DSC	Differential scanning calorimetry
DSF	Differential scanning fluorimetry
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
EPS	Ethylidene-blocked-4-nitrophenylmaltoheptaoside

F	Phenylalanine
4-FPBA	4-Formylphenylboronic acid
FAEO	Fatty alcohol ethoxylates
FAES	Fatty alcohol ether sulphates
FDA	Food and Drug Administration
FFF	Field-flow fractionation
FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
GA	Glutaraldehyde
Glu	Glutamic acid
GPC	Gel permeation chromatography
HEDP	1-hydroxyethane 1,1-diphosphonic acid
HES	Hydroxyethyl starch
HPLC	High performance liquid chromatography
His	Histidine
HmIm	2-methylimidazole
HRP	Horseradish peroxidase
IEX	Ion exchange chromatography
IRMOF	IsoReticular metal-organic framework
LAS	Linear alkylbenzene sulfonates
LCST	Lower critical solution temperature
LOM	Launder-O-meter
MALS	Multiangle light scattering
MIL	*Matériaux de l'Institut Lavoisier*
MOF	Metal-organic framework
mPEG	Methylated PEG
NHS	*N*-hydroxysuccinimide
NP	Nanoparticles
OTMS	Octadecyltrimethoxysilane
P	Proline
PCN	Porous coordination network
PCR	polymerase chain reaction
PEG	Polyethylene glycol
Phe	Phenylalanine
pI	Isoelectric point

PMSF	Phenylmethylsulphonyl fluoride
PNIPAm	Poly(*N*-isopropylacrylamide)
Pro	Proline
PS	Polystyrene
PVP	Polyvinylpyrrolidon
PXRD	Powder X-Ray diffraction
QELS	Quasi-elastic light scattering
RI	Refractive index
RP	Reversed phase
SDF	Standard detergent formulation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SEN	Single enzyme nanoparticle
Ser	Serine
Suc-AAPF-pNA	Suc-Ala-Ala-Pro-Phe-*para*-nitroaniline
TAED	Tetraacetylethylenediamine
TCA	Trichloroacetic acid
TEA	Triethanolamine
TEM	Transmission electron microscopy
TEOS	Tetraethyl orthosilicate
TEC	Triethyl citrate
TFA	Trifluoroacetic acid
TGA	Thermogravimetric analysis
TNBD	Trinitrobenzenesulfonic acid
UV	Ultraviolet
VIS	Visible
ZIF	Zeolitic imidazolate framework

9. List of Figures

10. List of Tables

11. Appendix

11.1. Reference Experiments

Respective nanoDSC thermograms of protease, α-amylase and lipase in phosphate buffer in a pH range of 3 to 11 (chapter 5.1.1.).

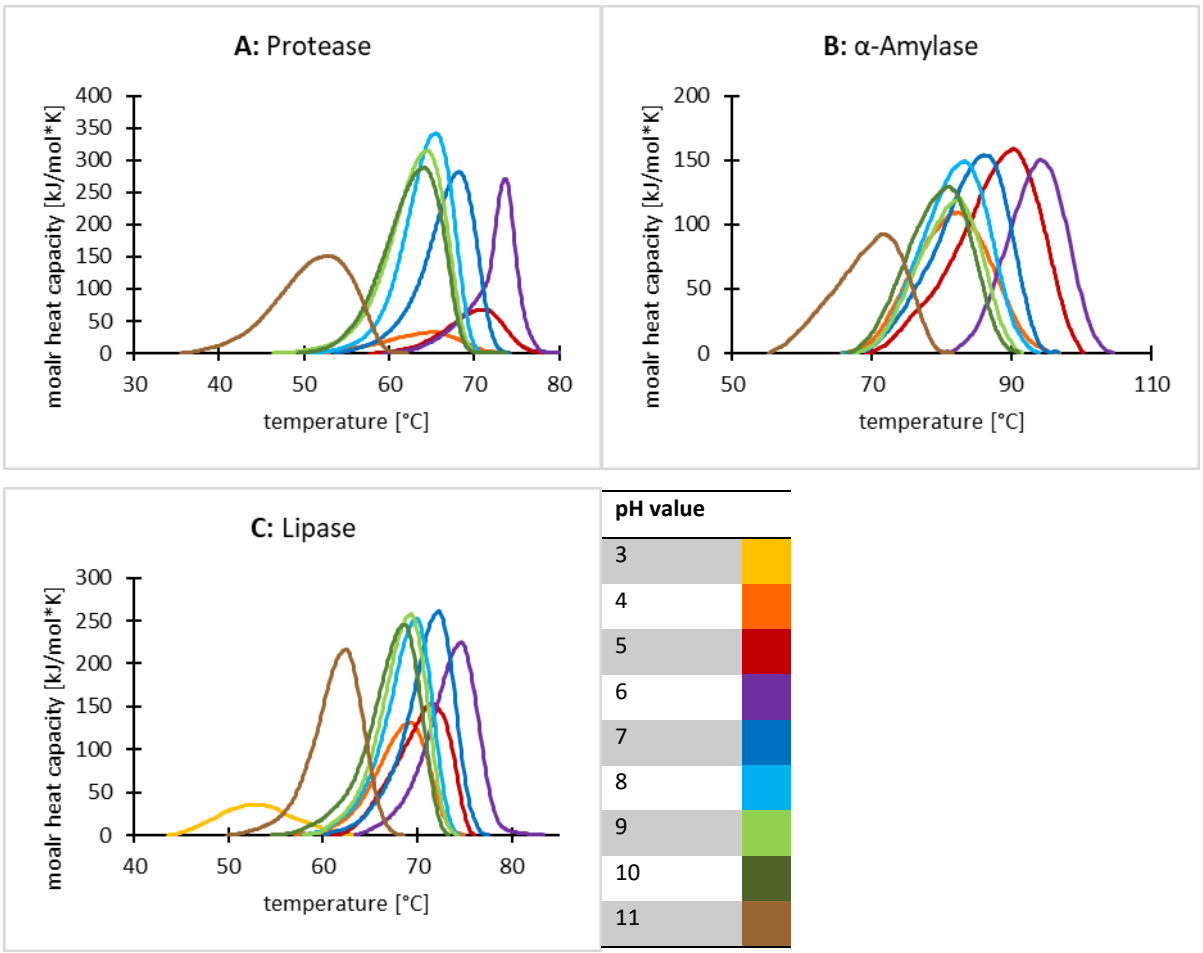


Figure 11-1: Thermograms showing thermal stability of protease, α-amylase and lipase in phosphate buffer (100 mM) in a pH range of 3 to 11.

Thermograms obtained by nanoDSC measurements of protease, α-amylase and lipase in water and a standard detergent formulation (chapter 5.1.2.).

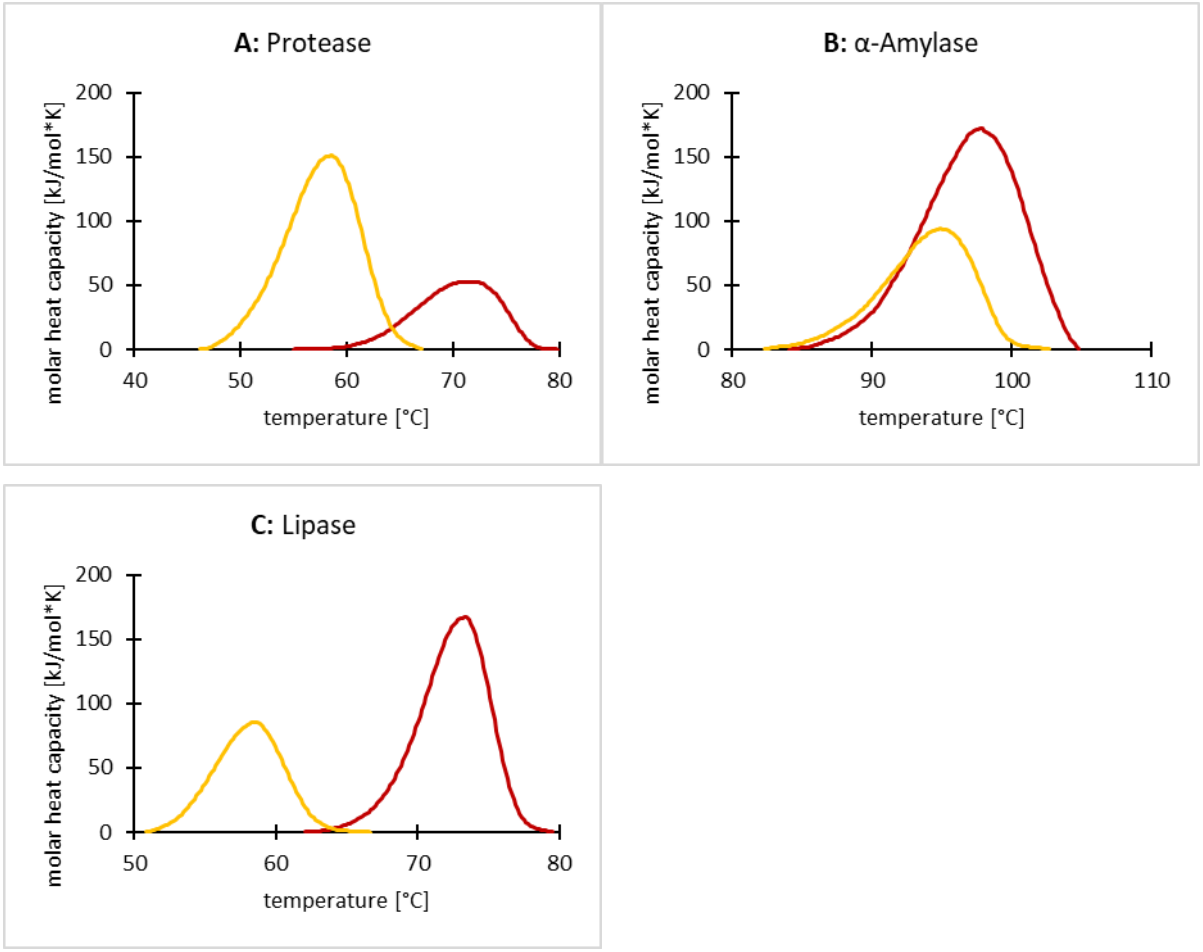


Figure 11-2: Thermograms of protease, α-amylase and lipase in water and in a standard detergent formulation measured by nanoDSC. ● standard detergent formulation ● water.

Respective nanoDSF thermograms of protease, α-amylase and lipase in phosphate buffer in a pH range of 3 to 11 (chapter 5.1.1.).

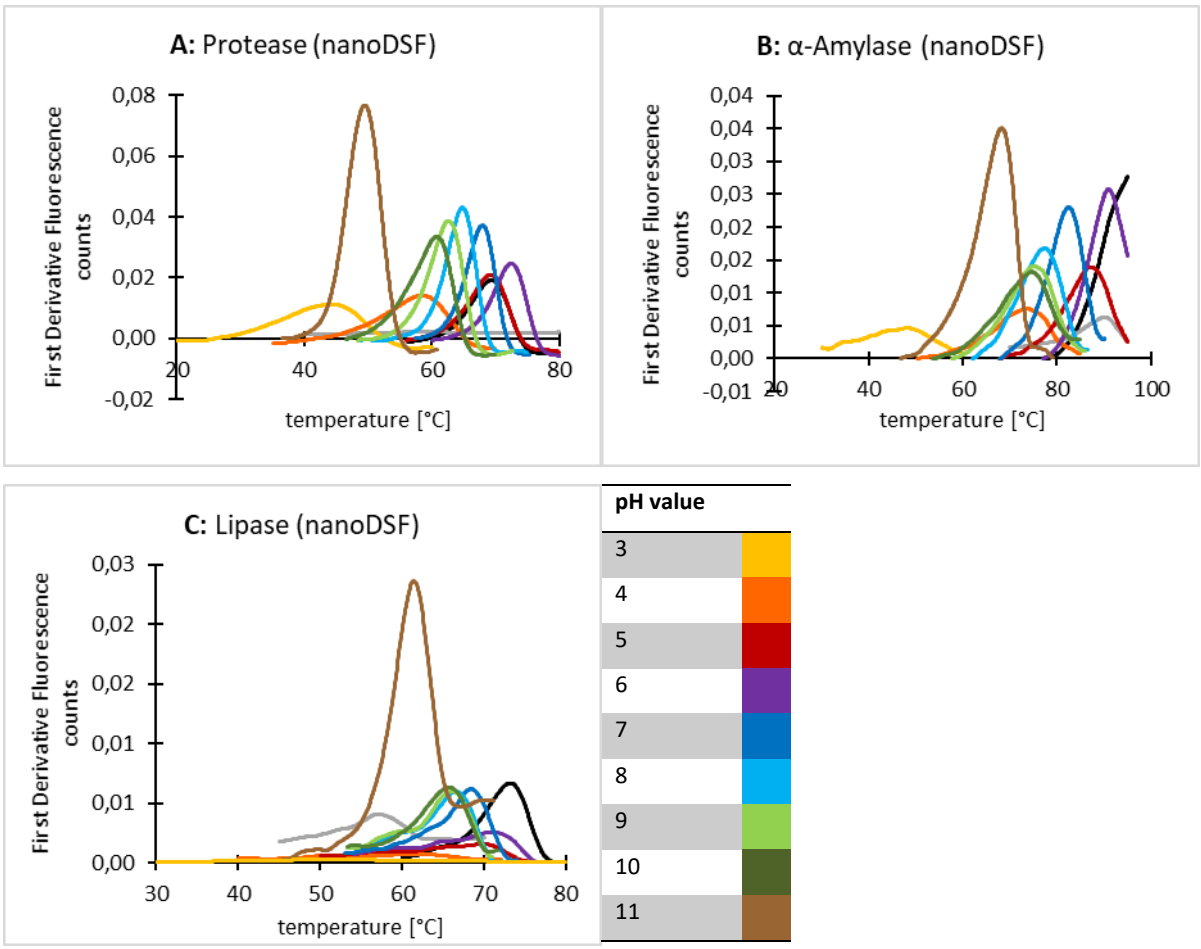


Figure 11-3: Thermograms showing thermal stability of protease, α-amylase and lipase in phosphate buffer (100 mM) in a pH range of 3 to 11.

Thermograms obtained by nanoDSF measurements of protease, α-amylase and lipase in water and a standard detergent formulation (chapter 5.1.1.).

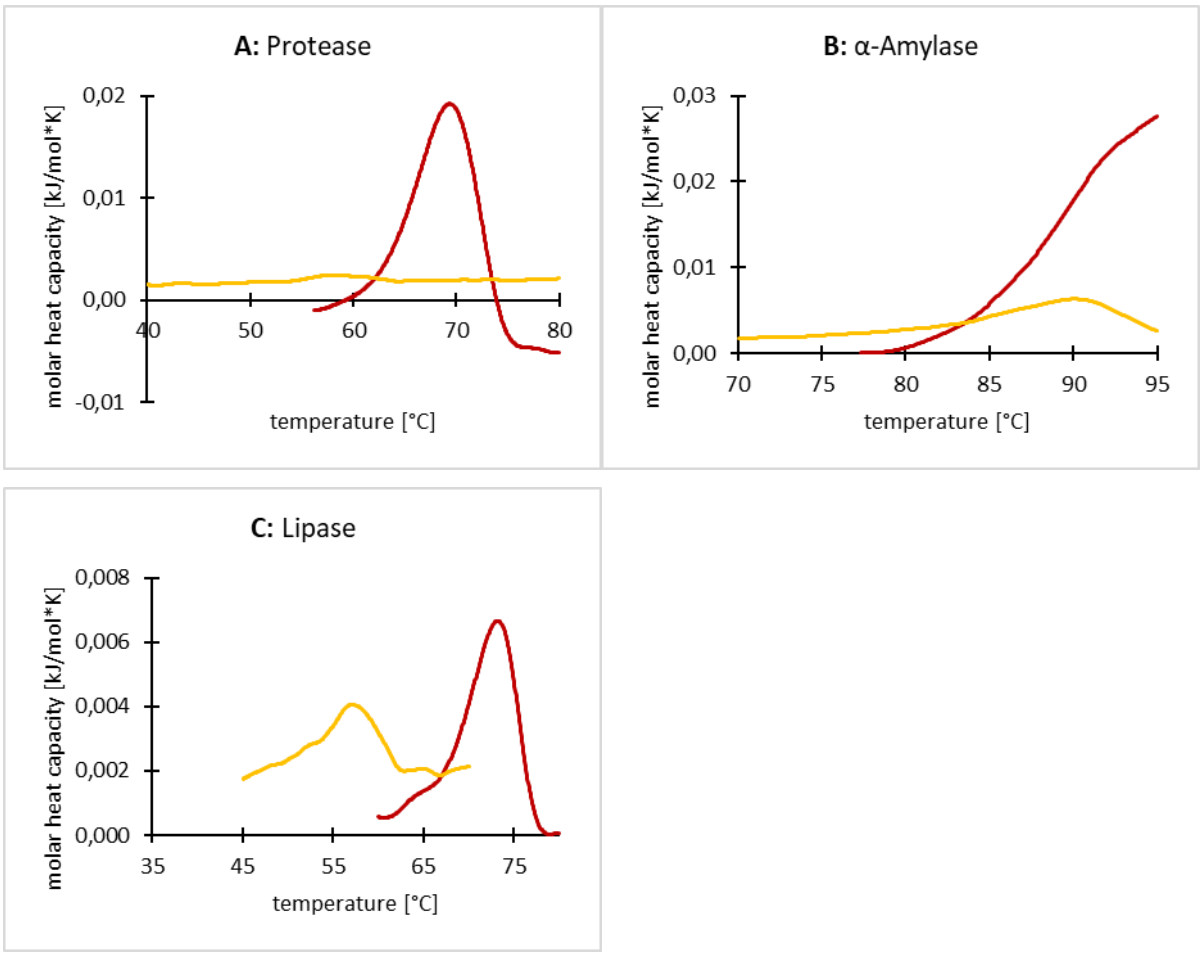


Figure 11-4: Thermograms of protease, α-amylase and lipase in water and in a standard detergent formulation measured by nanoDSF. ● standard detergent formulation ● water.

Respective DSF assay thermograms of α-amylase and lipase in phosphate buffer in a pH range of 3 to 11 (chapter 5.1.1.).

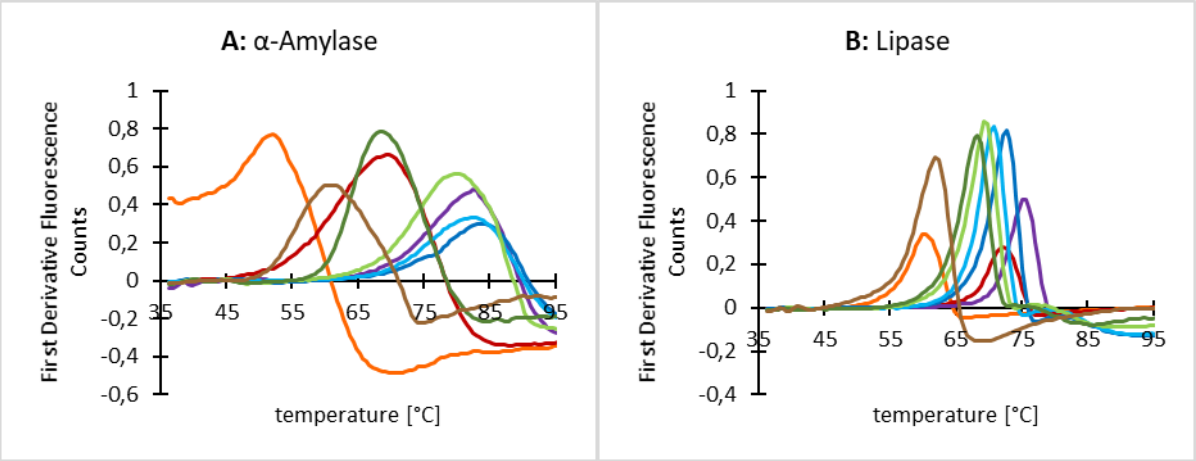


Figure 11-5: Thermograms showing thermal stability of α-amylase and lipase in phosphate buffer (100 mM) in a pH range of 3 to 11.

Thermograms obtained by nanoDSF measurements of α-amylase and lipase in water (chapter 5.1.1.).

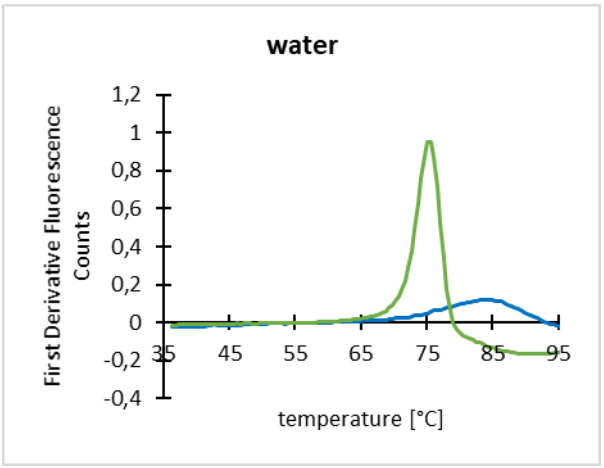


Figure 11-6: Thermograms of α-amylase and lipase in water measured by DSF assay. ● α-amylase ● lipase.

Stability of protease, α-amylase and lipase in the presence of chelators – 20 mM EDTA, 40 mM HEDP and 200 mM Citrate (chapter 5.1.3.). Enzyme activity measured in storage tests and thermal stability determined using nanoDSC.

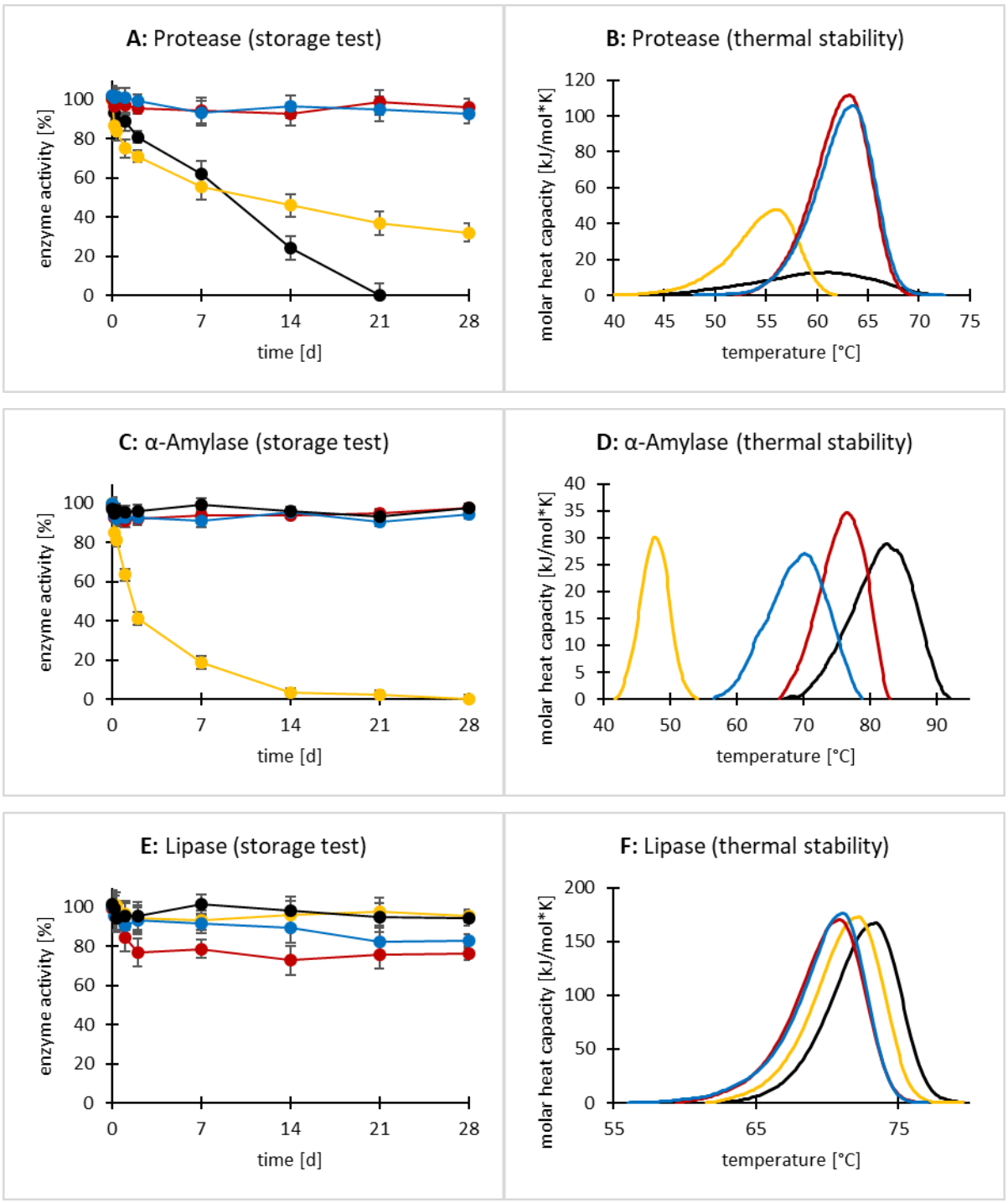
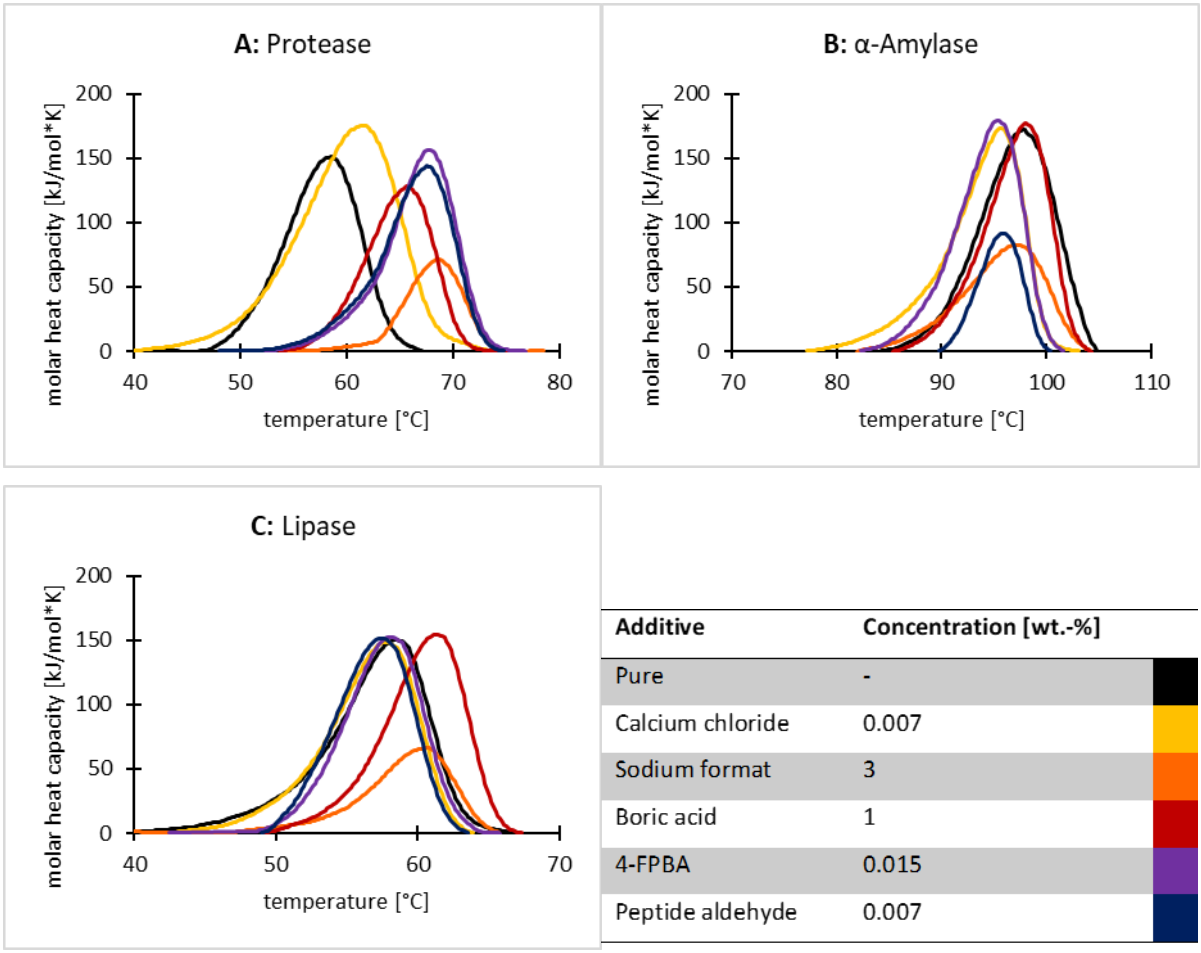


Figure 11-7: Stability of protease, α-amylase and lipase in the presence of EDTA (20 mM), HEDP (40 mM) and citrate (200 mM) in aqueous solutions. Storage tests A (protease), C (α-amylase) and E (Lipase). Thermograms regarding thermal stability B (protease), D (α-amylase) and F (Lipase). ● pure enzyme ● EDTA ● HEDP ● citrate. Connecting lines are only guide to the eye.

11.2. Small Molecules

Thermograms of thermal stability measurements in presence of the established small molecules (chapter 5.2.1.).



Additive	Concentration [wt.-%]
Pure	-
Calcium chloride	0.007
Sodium format	3
Boric acid	1
4-FPBA	0.015
Peptide aldehyde	0.007

Figure 11-8: Enzyme stability in a standard detergent formulation with various established small molecules as additives. The stability is determined by measurement of the thermal stability using nanoDSC.

Thermograms of thermal stability experiments used for concentration screening of the additives acetylcholine, citric acid choline ester, triethyl citrate and acetyl triethyl citrate (chapter 5.2.2.).

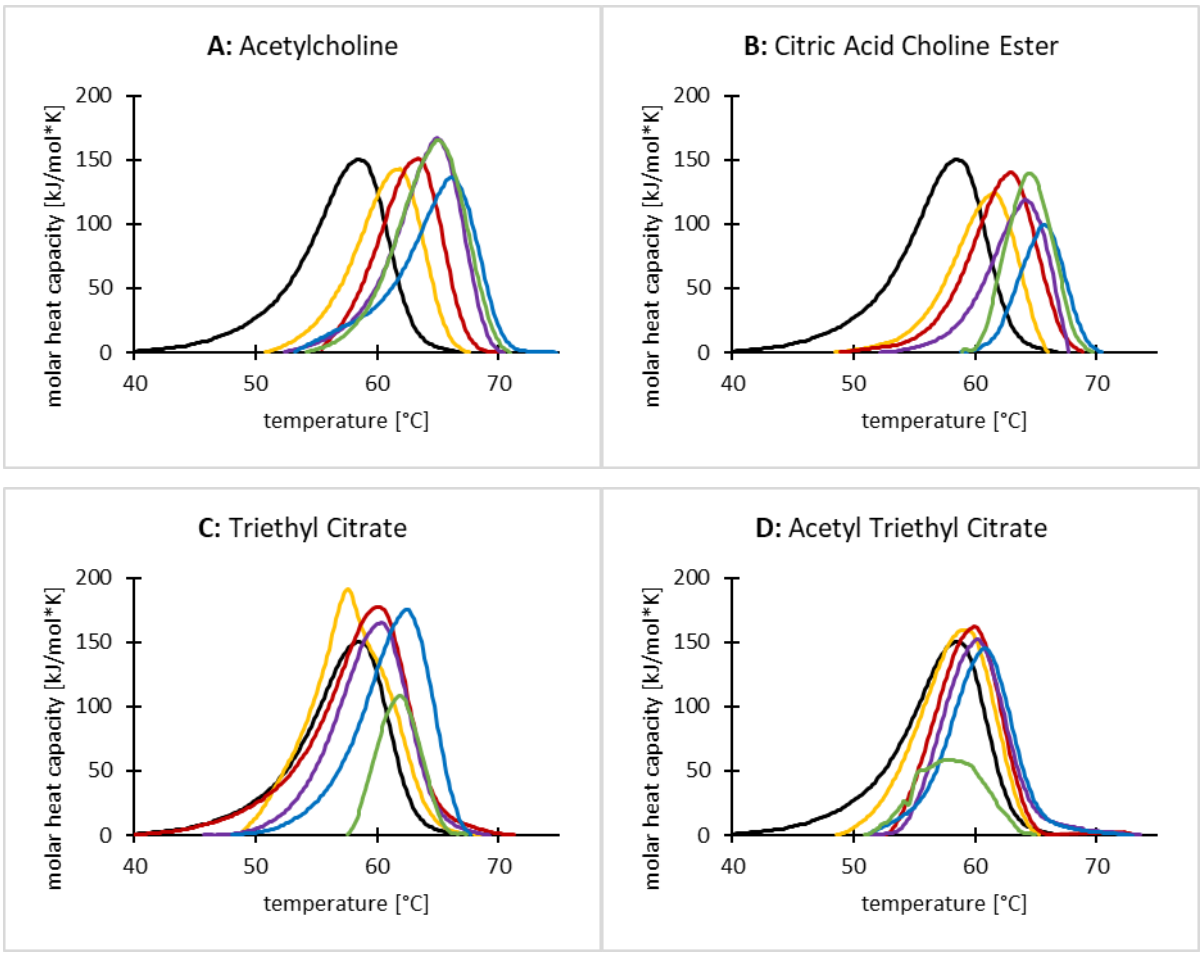


Figure 11-9: Thermograms obtained from concentration screening of lipase thermal stability with the addition of acetylcholine (A), citric acid choline ester (B), triethyl citrate (C) and acetyl triethyl citrate (D). Measurements are conducted in a standard detergent formulation. ● pure enzyme ● 0.5wt.-% additive ● 0.9wt.-% additive ● 1.5wt.-% additive ● 2.0wt.-% additive ● 4.0wt.-% additive.

Thermograms of thermal stability experiments with protease and α-amylase in presence of acetylcholine, citric acid choline ester, triethyl citrate and acetyl triethyl citrate (2wt.-%; chapter 5.2.3.).

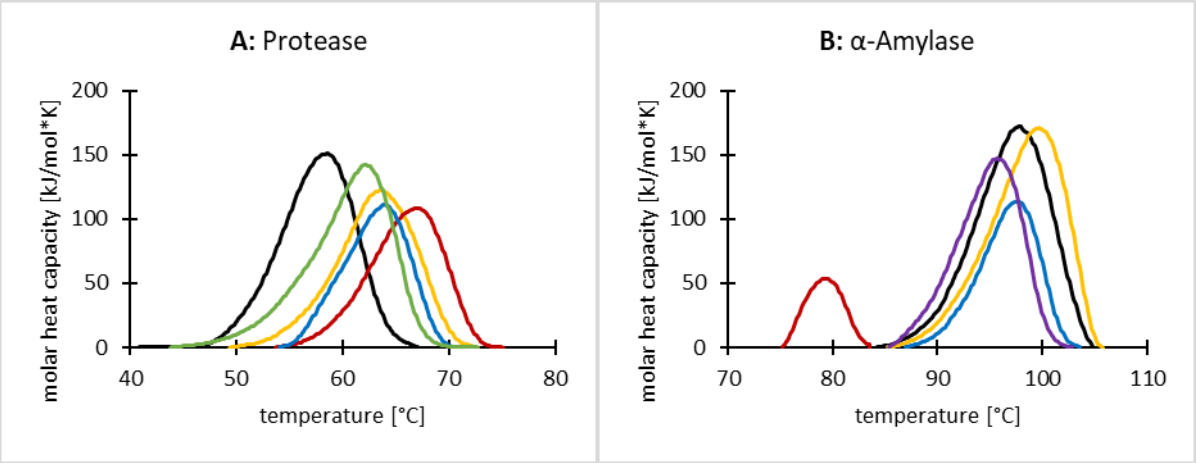


Figure 11-10: Thermograms of thermal stability experiments of protease and α-amylase with the addition of ACh, CACE, TEC and ATEC in a standard detergent formulation. ● pure enzyme ● CACE ● ACh ● TEC ● ATEC.

Thermograms of thermal stability experiments with lipase in the presence of acetylcholine, citric acid choline ester, triethyl citrate and acetyl triethyl citrate (2wt.-%) after 28 days storage and direct measurement of the decomposed ester compounds (chapter 5.2.3.).

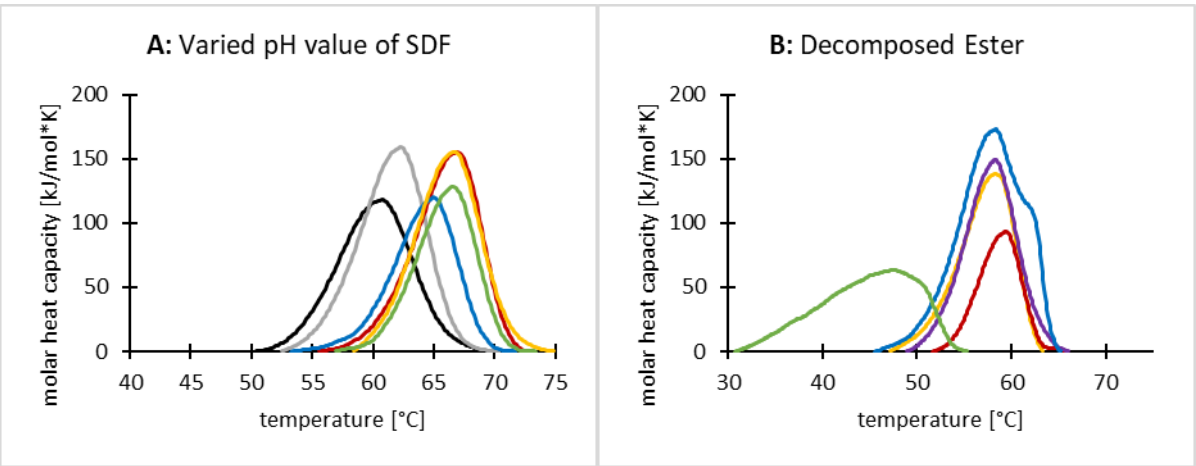


Figure 11-11: Thermograms regarding the thermal stability of lipase in a standard detergent formulation. A: ● pure enzyme (pH=5.5) ● pure enzyme (pH=6.5) ● CACE ● ACh ● TEC ● ATEC. SDF solutions measured directly, additive solutions after 28 d storage at 37 °C. B: Hydrolyzed ester compounds. ● 1wt.-% choline ● 0.5wt.-% choline ● 0.4wt.-% citric acid ● 0.2wt.-% ethanol ● 0.4wt.-% acetic acid.

11.3. Enzyme-Polymer Conjugates

Reaction control and determination of conversion rate of the enzyme-polymer conjugate synthesis using SDS-PAGE and HPLC (chapter 5.3.1.).

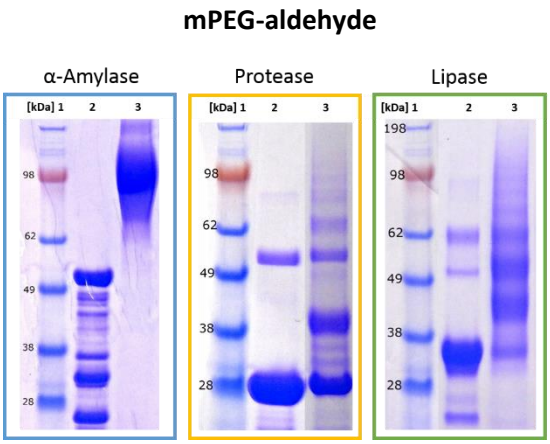


Figure 11-12: SDS-PAGE of non-PEGylated (lane 2), PEGylated α-amylase, protease and lipase (lane 3) and marker (lane 1). PEGylation was performed with mPEG-aldehyde. This figure is obtained from a publication (Macromol. Biosci., **2018**, *18*, 1800095) with permission from Wiley-VCH 2019.

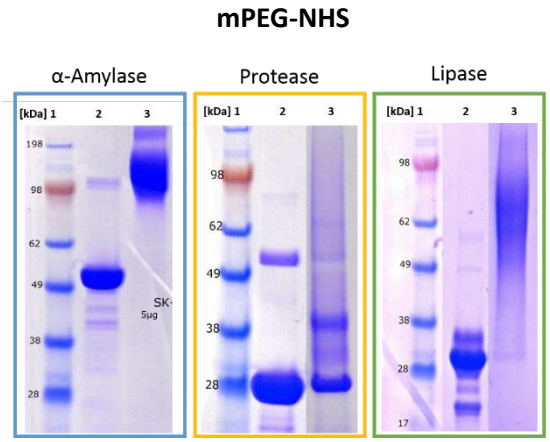


Figure 11-13: SDS-PAGE of non-PEGylated (lane 2), PEGylated α-amylase, protease and lipase (lane 3) and marker (lane 1). PEGylation was performed with mPEG-NHS. This figure is obtained from a publication (Macromol. Biosci., **2018**, *18*, 1800095) with permission from Wiley-VCH 2019.

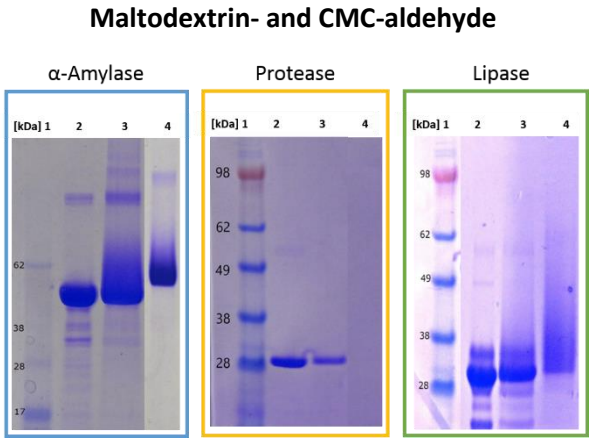


Figure 11-14: SDS-PAGE of non-conjugated (lane 2), conjugated α-amylase, protease and lipase (lane 3 and 4) and marker (lane 1). Conjugation was performed with CMC- (lane 3) and maltodextrin-aldehyde (lane 4). This figure is obtained from a publication (Macromol. Biosci., **2018**, *18*, 1800095) with permission from Wiley-VCH 2019.

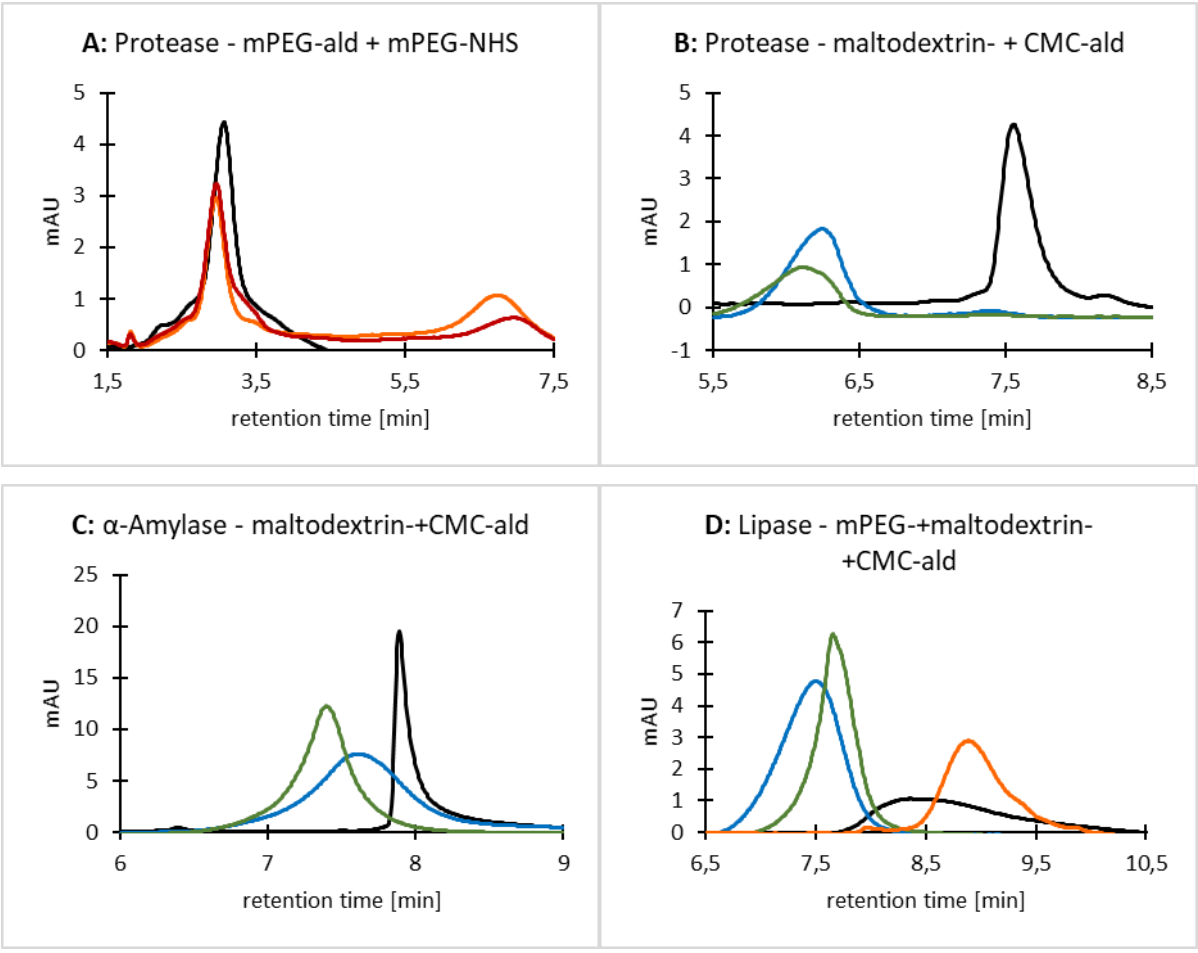


Figure 11-15: HPLC chromatogram of non-conjugated and conjugated enzymes. ● pure enzyme ● conjugate mPEG-ald ● conjugate mPEG-NHS ● conjugate maltodextrin-ald ● conjugate CMC-ald. This figure is obtained from a publication (Macromol. Biosci., **2018**, *18*, 1800095) with permission from Wiley-VCH 2019.

The number of polymers covalently linked to one enzyme molecule is determined using asymmetrical-flow field-flow fractionation (AF4; chapter 5.3.2.).

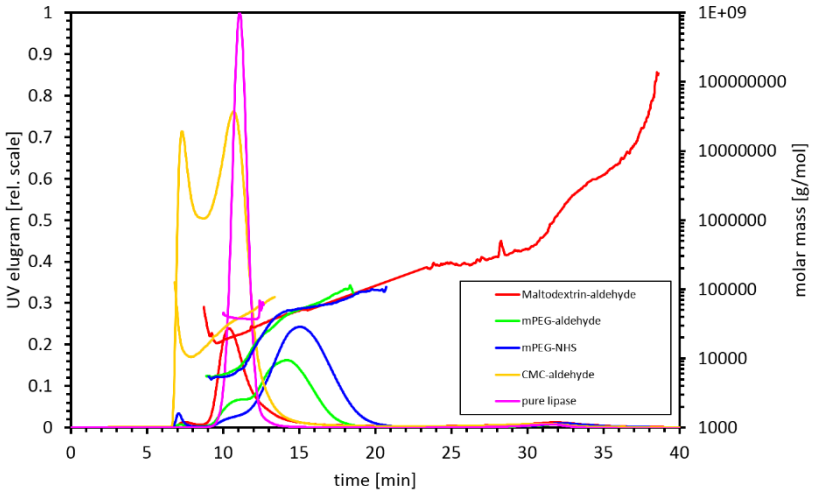


Figure 11-16: UV elugram and results of measurements on the determination of molar mass of conjugated and non-conjugated lipase. This figure is obtained from a publication (Macromol. Biosci., **2018**, *18*, 1800095) with permission from Wiley-VCH 2019.

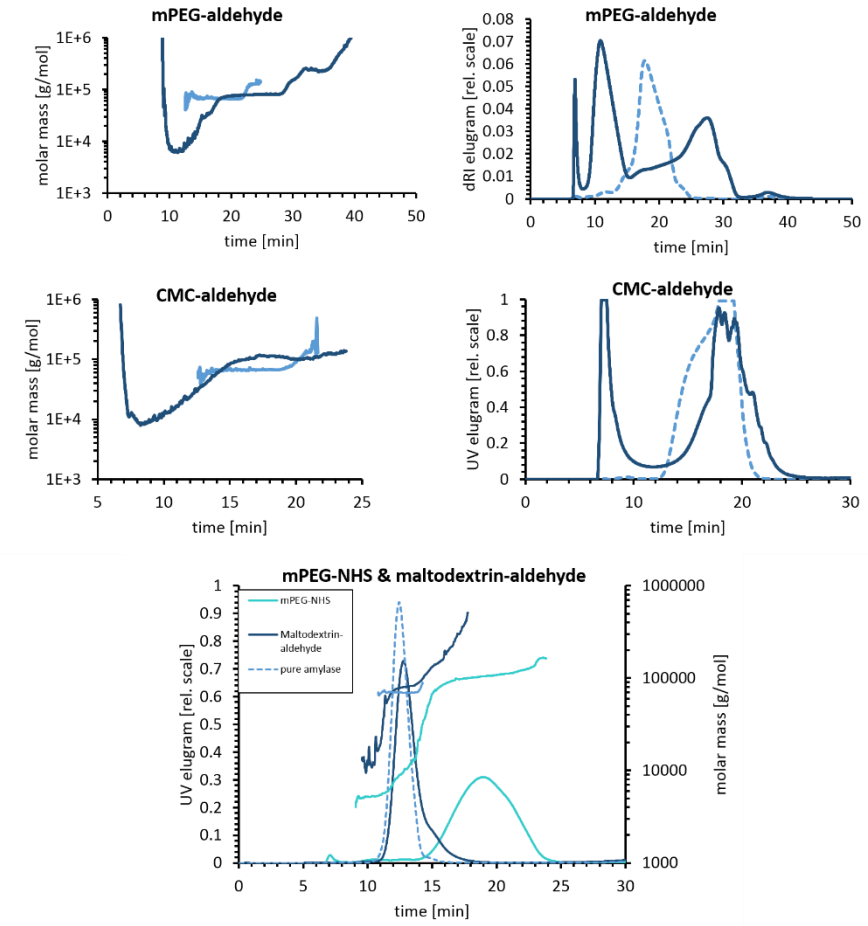


Figure 11-17: UV/ dIR elugram and results of measurements on the determination of molar mass of conjugated and non-conjugated α amylase. Pure enzyme with dotted light lines and conjugates with solid dark lines. This figure is obtained from a publication (Macromol. Biosci., **2018**, *18*, 1800095) with permission from Wiley-VCH 2019.

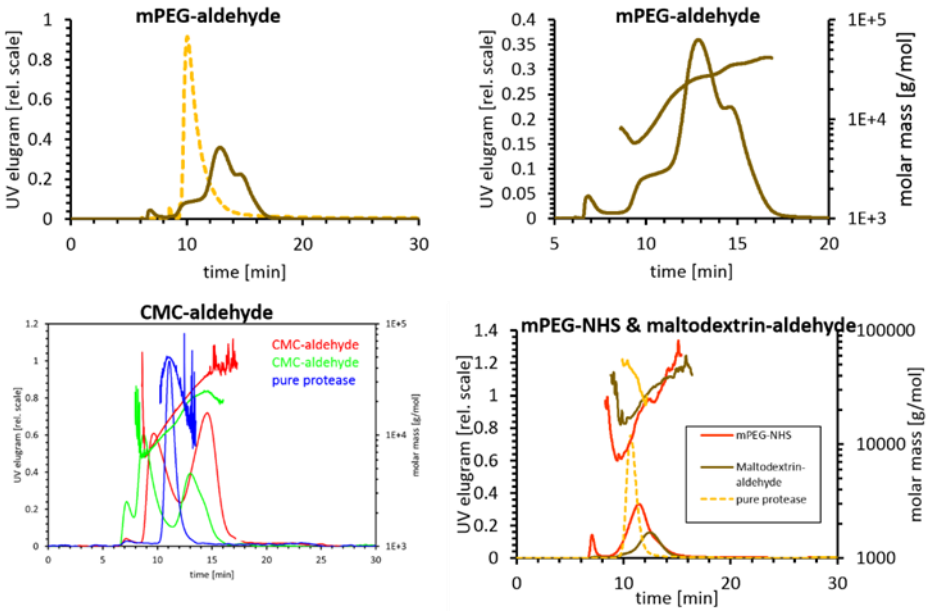


Figure 11-18: UV elugram and results of measurements on the determination of molar mass of conjugated and non-conjugated protease. Pure enzyme with dotted light lines and conjugates with solid dark lines. This figure is obtained from a publication (Macromol. Biosci., **2018**, *18*, 1800095) with permission from Wiley-VCH 2019.

Thermal stability of enzyme-polymer conjugates is determined using nanoDSC. Thermograms are shown here (chapter 5.3.2.).

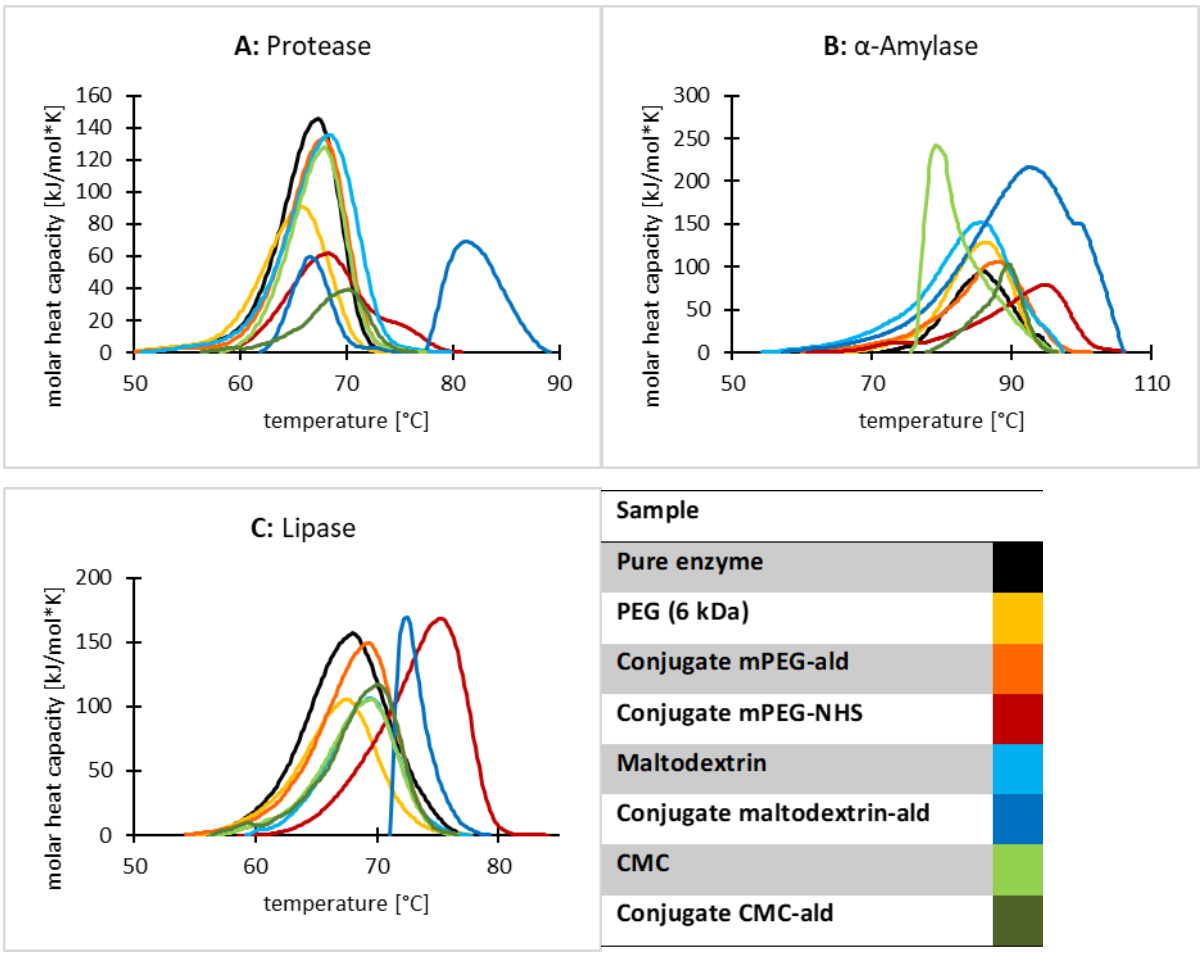


Figure 11-19: Thermograms for determination of thermal stability of enzyme-polymer conjugates. A: protease; B: α-amylase; C: lipase.

11.4. Mesoporous Silica Nanoparticles

Determination of pore size of TEOS-OTMS particles before and after calcination by BET (chapter 5.4.1).

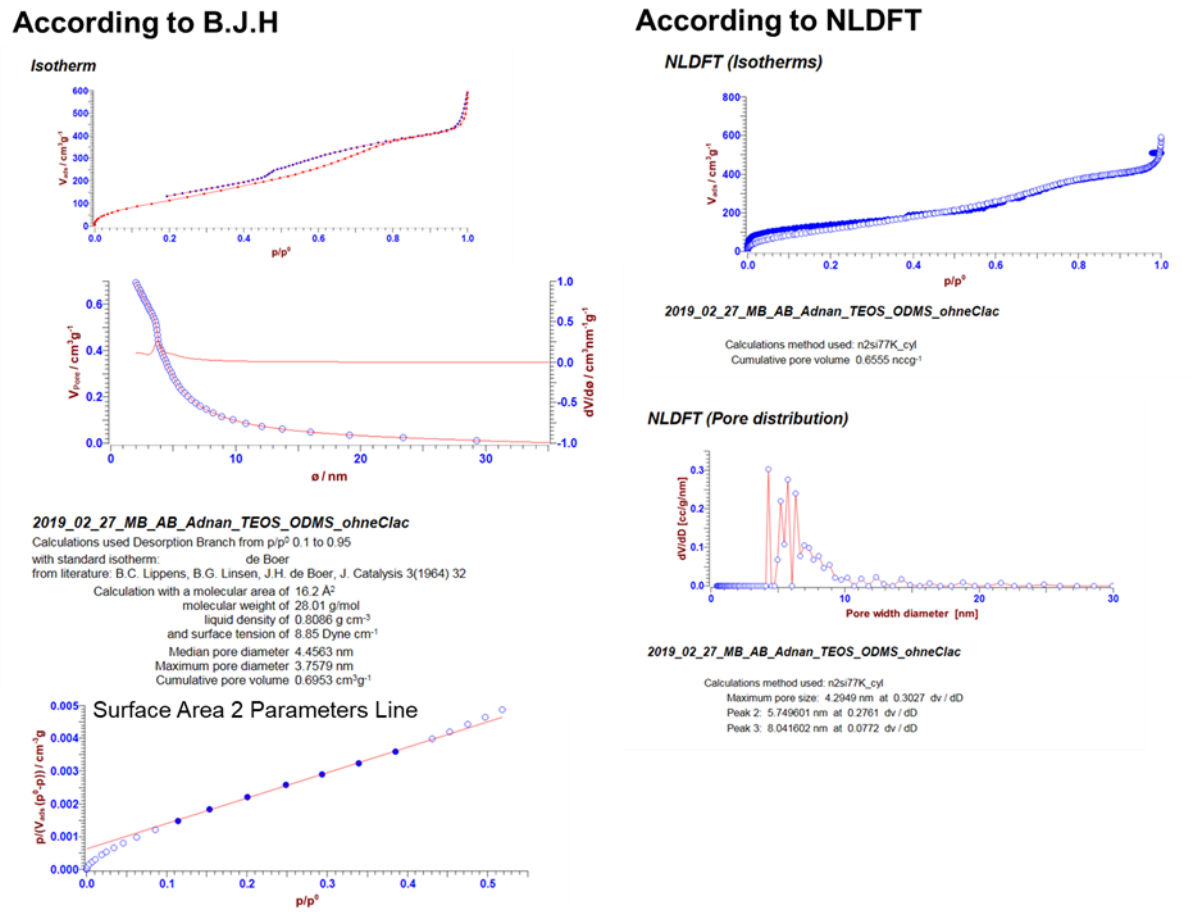


Figure 11-20: Results BET before calcination of TEOS-OTMS particles. Experiments regarding nitrogen sorption are conducted by Martin Brodrecht research group of Professor Gerd Buntkowsky, TU Darmstadt.

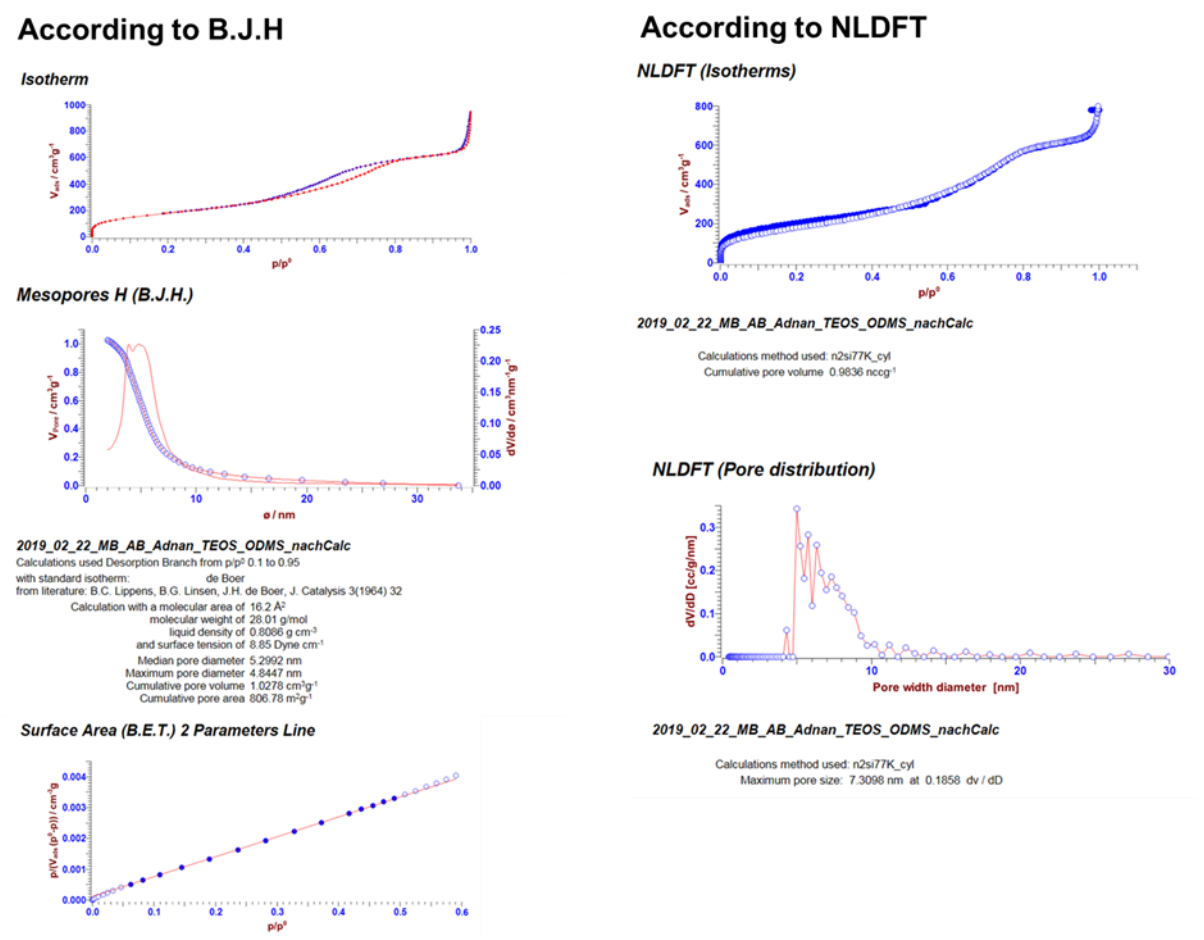


Figure 11-21: Results BET after calcination of TEOS-OTMS particles. Experiments regarding nitrogen sorption are conducted by Martin Brodrecht research group of Professor Gerd Buntkowsky, TU Darmstadt.

Results of ATR-IR measurements regarding particle stability at alkaline pH values (chapter 5.4.1.).

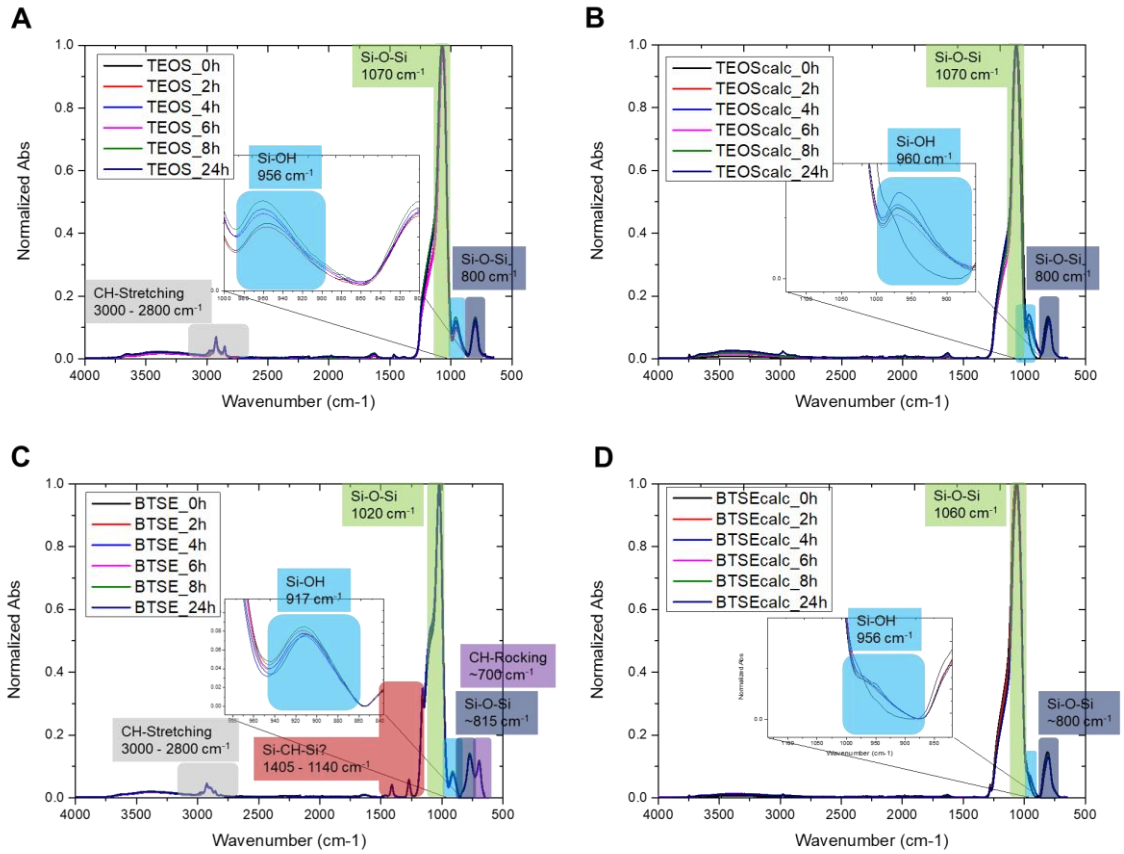


Figure 11-22: ATR-IR measurements regarding stability at alkaline pH values. A: TEOS-OTMS particles before calcination. B: TEOS-OTMS particles after calcination. C: BTSE-OTMS particles before calcination. D: BTSE-OTMS particles after calcination.

11.5. Metal-Organic Frameworks

Protease activity in presence of metal-organic frameworks containing lipase in a storage test at 22 °C in water (chapter 5.5.2).

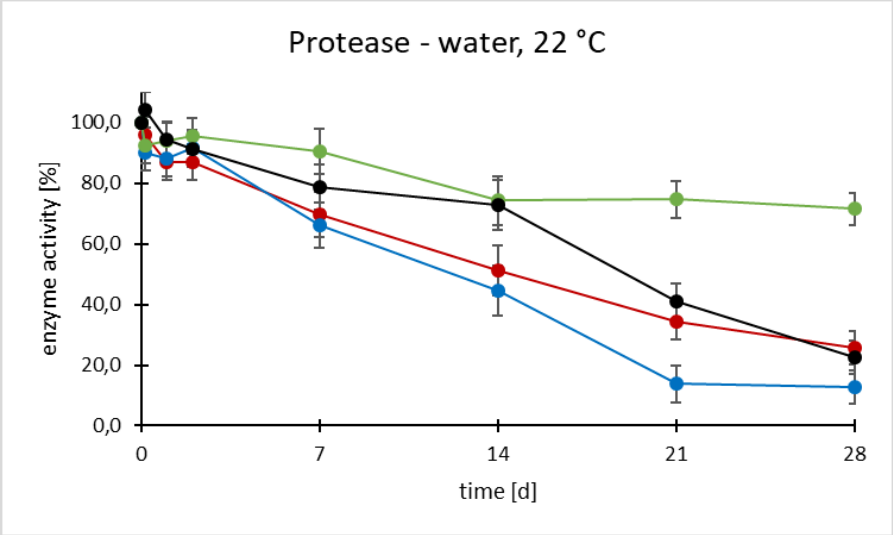


Figure 11-23: Protease activity in water at 22 °C in presence of metal-organic frameworks. ● protease + ZIF-8⊂lipase ● protease + crystalline MOF-74⊂lipase ● protease lipase ● pure protease. Connecting lines are only guide to the eye.

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Erklärung

Ich erkläre hiermit, dass ich meine Dissertation selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt und noch keinen Promotionsversuch unternommen habe.

_____

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Erklärung der Übereinstimmung

Ich erkläre hiermit, dass die elektronische Version der Doktorarbeit mit der schriftlichen Version übereinstimmt. Die elektronische Version liegt dem Prüfungssekretariat vor.

_____

Sonja Kübelbeck